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ABC efflux transporters and its relevance on the bioavailability and detoxification of polycyclic aromatic hydrocarbons (PAHs)

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"Each problem that I solved became a rule, which served afterwards to solve other problems."

René Descartes

À minha família...

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De acordo com o disposto no nº 2, alínea a, do artigo 31º do decreto lei nº 230/2009, utilizaram-se neste trabalho resultados já publicados ou em vias de publicação, que a seguir se enumeram:

1. **Costa, J.**, Ferreira, M., Rey-Salgueiro, L., Reis-Henriques, M.A., 2011. Comparision of the waterborne and dietary routes of exposure on the effects of Benzo(a)pyrene on biotransformation pathways in Nile tilapia (*Oreochromis niloticus*). Chemosphere 84, 1452-1460.

2. Rey-Salgueiro, L., **Costa, J.**, Ferreira, M., Reis-Henriques, M.A., 2011. Evaluation of 3-hydroxy-benzo[a]pyrene levels in Nile tilapia (*Oreochromis niloticus*) after waterborne exposure to Benzo[a]pyrene. Toxicological & Environmental Chemistry 93, 2040-2054.

3. **Costa, J.**, Reis-Henriques, M.A., Castro, L.F.C., Ferreira, M., 2012. Gene expression analysis of ABC efflux transporters, CYP1A and GSTα in Nile tilapia after exposure to benzo(a)pyrene. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 155, 469-482.

4. **Costa, J.**, Reis-Henriques, M.A., Wilson, J.M., Ferreira, M. 2012. Tissue distribution and response patterns of Pgp and CYP1A proteins in Nile tilapia (*Oreochromis niloticus*) after waterborne exposure to Benzo(a)pyrene (BaP). Submitted for publication in Toxicology and applied pharmacology.

5. **Costa, J.**, Reis-Henriques, M.A., Castro, L.F.C., Ferreira, M. 2012. ABC transporters, CYP1A and GSTα gene transcription patterns in developing stages of the Nile tilapia (*Oreochromis niloticus*). Gene. DOI: 10.1016/j.gene.2012.06.092

Abstract

The detoxification mechanisms of living organisms are crucial for their normal growth and survival. Aquatic organisms are in face of a particularly difficult task since their habitats have long been the last receptacle for several different types of pollutant compounds, mainly resultant from anthropogenic sources. One of the best studied mechanisms of detoxification is the biotransformation of the xenobiotic compounds into more easily excreted forms, in a two phase (I and II) process of enzymatic reactions. In recent years, the cellular efflux of pollutants and/or their metabolites by some proteins of the ATP-Binding Cassette Superfamily (ABC) has also been considered as an important defence mechanism. ABC proteins were first recognized for their role in multidrug resistance (MDR) in chemotherapeutic treatments, which is a major impediment for the successful treatment of many forms of malignant tumors in humans. These proteins, found to be highly conserved throughout vertebrate species, were later related to cellular detoxification and accounted as responsible for protecting aquatic organisms from xenobiotic insults in the so-called multixenobiotic resistance mechanism (MXR). Moreover, recent studies in different mammalian models have provided some evidence that, ABC efflux transporters and biotransformation enzymes, act in coordination, resulting in an effective global mechanism of cellular detoxification. Hence, the full knowledge of the functionality of these detoxification processes is needed, both for environmental as for human health.

Nevertheless, despite the importance of these mechanisms, and although ABC efflux transporters have been identified in several fish species, information regarding their mode of action is still scarce, namely on the integration of these transporters in the detoxification pathway, and on the cooperation with the biotransformation enzymes. This study was delineated to fill these gaps, by assessing the responses of the three phases of cellular detoxification after *in vivo* exposures of a fresh water species (Nile tilapia, *Oreochromis niloticus*) to a highly toxic polycyclic aromatic hydrocarbon (PAH), Benzo(a)pyrene (BaP).

The biotransformation of BaP by phase I and phase II enzymes was first investigated in Nile tilapia in order to determine the effectiveness of their biotransformation system. Based on the obtained results, we concluded that the pathway of detoxification was dependent on the route of exposure to the contaminant. Different barrier tissues were primarily involved in BaP metabolism whether the exposure occurred through the water

(liver, gills and intestine) or through the ingestion of contaminated food (intestine). These findings are of sovereign value for environmental risk assessment studies since they imply that, besides liver, the study of extra-hepatic tissues, as gills and intestine, may provide valuable information on the major sources of the contamination in the field. The phase I enzyme cytochrome P4501A (CYP1A) was demonstrated to display a paramount role on the metabolism of BaP in the different barrier tissues (liver, gills and intestine), at gene, protein and activity levels. Moreover, phase II enzymes (Glutathione-S-Transferases, GSTs and UDP-glucuronyl transferases, UGTs) were shown to be capable of neutralizing the toxic phase I metabolites, including 3-OH-BaP, since since the majority of BaP metabolites found in bile and plasma of Nile tilapia were phase II conjugates. Therefore, our findings have shown that Nile tilapia has a well-developed biotransformation system, capable of providing an effective detoxification pathway to BaP, despite the main route of exposure.

In order to assess the cooperation between ABC transporter genes and drug metabolizing enzymes, ecotoxicologically relevant ABC efflux transporter genes (*ABCB1b, ABCB11, ABCC1, ABCC2 and ABCG2*) were isolated, and their modulation, along with phase I and II biotransformation enzymes, to BaP was evaluated after dietary and waterborne exposure routes in Nile tilapia. Although *ABCB1b* was found not to be altered by BaP exposure (indicating that BaP should not be a substrate for Pgp), both at gene at protein level, *ABCC2* and *ABCG2a* could be related to the efflux of phase II metabolites, and they probably display major roles in gills and proximal intestine of fish. Thus, our results clearly reflected the cooperation between efflux transporters and biotransformation enzymes in BaP detoxification in Nile tilapia. Evidence of the crucial role of these transporters and biotransformation enzymes in the protection of organisms were further provided, as they were found to be expressed since the onset of Nile tilapia embryos development. *ABCB1b*, *ABCC1*, *CYP1A* and *GSTα* genes were maternally transmitted to Nile tilapia, which suggests that they are essential for the embryos survival. *ABCB11, ABCC2* and *ABCG2* gene transcription was initiated later in the development, but preceding the highly sensitive period of hatching, indicating that the higher defence levels that may be necessary at this stage can be assured by these particular proteins. These findings are, therefore, important to support the highly valuable role of ABC transporters and biotransformation enzymes in the protection against xenobiotics in fish and reflect that these groups of proteins cooperate to provide aquatic organisms with an effective mechanism of cellular detoxification.

Resumo

Os mecanismos de destoxificação de todos os seres vivos são cruciais para o seu normal desenvolvimento e sobrevivência. Os organismos aquáticos estão perante uma tarefa particularmente difícil uma vez que, ao longo dos anos, o seu habitat tem sido o recipiente final para um vasto conjunto de poluentes, principalmente resultantes de fontes antropogénicas. Um dos mecanismos de destoxificação mais estudado é a biotransformação de compostos xenobióticos, em metabolitos mais facilmente excretáveis, através de reacções enzimáticas em duas fases (fase I e fase II). Recentemente, o efluxo cellular de poluentes e/ou dos seus metabolitos, por acção de proteínas pertencentes à superfamília ATP-Binding Cassette (ABC), tem sido também considerado um importante mecanismo de defesa. As proteínas ABC foram inicialmente reconhecidas pelo seu papel na resistência a multi-drogas (MDR) durante tratatamentos quimioterapêuticos, factor que tem sido responsável pela diminuição da taxa de sucesso no que respeita ao tratamento de várias formas de tumores malignos em humanos. Estas proteínas, que são muito conservadas ao longo das várias espécies de vertebrados, foram, mais tarde, relacionadas com a destoxificação celular, e indicadas como responsáveis pela proteção de organismos aquáticos contra danos causados por xenobióticos, num mecanismo de resistência multi-xenobiótica (MXR). Adicionalmente, estudos recentes em diferentes modelos animais de mamíferos têm fornecido evidências de que os transportadores ABC e as enzimas de biotransformação actuam em cooperação, formando um mecanismo efectivo de destoxificação celular. Desta forma, o conhecimento da funcionalidade destes mecanismos de destoxificação torna-se necessário, tanto para a saúde ambiental como para a saúde humana.

No entanto, apesar da importância destes mecanismos, e embora os transportadores de efluxo ABC tenham vindo a ser identificados em diferentes espécies de peixes, há ainda pouca informação sobre o seu mecanismo de acção, nomeadamente na integração destes transportadores nas vias de destoxificação, e na cooperação com as enzimas de biotransformaçao. Este estudo foi delineado para preencher essas lacunas, através da avaliação das respostas das três fases de destoxificação celular após exposições *in vivo*, de uma espécie de água doce (Tilápia do Nilo, *Oreochromis niloticus*), a um hidrocarboneto aromático policíclico (PAH) com propriedades altamente tóxicas, o Benzo(a)pireno (BaP)

A biotransformação do BaP pelas enzimas de fase I e fase II foi investigada na tilápia do Nilo por forma a determinar a eficácia mecanismo de biotransformação desta

espécie. Com base nos resultados obtidos, concluímos que a via de destoxificação está dependente da via de exposição ao contaminante. Diferentes tecidos barreira estiveram primeiramente envolvidos no metabolismo do BaP consoante a exposição ocorreu através da água (fígado, brânquia e intestino), ou através da ingestão de alimento contaminado (intestino). Estes resultados são importantes em estudos de avaliação de risco ambiental uma vez que implicam que, para além do fígado, o estudo de tecidos extra-hepáticos, tais como a brânquia e o intestino, pode fornecer informação de valor sobre as fontes maioritárias de contaminação ambiental. A enzima de fase I, Citocromo P4501A (CYP1A), demonstrou desempenhar um importante papel no metabolismo do BaP nos diferentes tecidos barreira (fígado, brânquia e intestino), ao nível do gene, da proteína e da actividade catalítica. Adicionalmente, as enzimas de fase II (Glutationa-STransferases, GSTs e UDP-Glucoronil transferases, UGTs) mostraram ser capazes de neutralizar os metabolitos tóxicos de fase I, incluindo o 3-OH-BaP, visto que a maioria dos metabolitos de BaP encontrados na bílis e no plasma eram conjugados da fase II. Assim, foi possível demonstrar que a tilápia do Nilo possui um sistema de biotransformação bem desenvolvido, capaz de fornecer uma via de destoxificação efectiva para o BaP, independentemente da via de exposição.

De forma a avaliar a cooperação entre os transportadores ABC e as enzimas de biotransformação, os genes para transportadores ABC com relevância ecotoxicológica (*ABCB1b, ABCB11, ABCC1, ABCC2 and ABCG2a*) foram isolados e a sua modulação, juntamente com a das enzimas de biotransformação de fases I e II, foi avaliada após exposição ao BaP pela água e pela dieta na tilápia do Nilo. Apesar de a expressão génica do *ABCB1b* não se ter alterado após exposição ao BaP, tanto a nível do gene como da proteína (indicando que, provavelmente, o BaP não é um substrato para a Pgp), o aumento da expressão génica de *ABCC2* e *ABCG2a* pode ser relacionado com o efluxo de metabolitos de fase II, indicando que, provavelmente, estes transportadores desempenham importantes papéis na brânquia e intestino dos peixes. Estes resultados reflectem claramente a cooperação entre os transportadores de efluxo e as enzimas de biotransformação na destoxificação do BaP na tilápia do Nilo.

Indicações adicionais sobre o papel crucial destes transportadores e das enzimas de biotransformação na protecção dos organismos foram fornecidas, já que os genes que codificam para estas proteínas são expressos desde o início do desenvolvimento embrionário da tilápia do Nilo. Os genes de *ABCB1b*, *ABCC1* e *CYP1A* e *GSTα* são transmitidos maternalmente, sugerindo que serão essenciais para a sobrevivência dos embriões. A transcrição génica de *ABCB11, ABCC2* e *ABCG2* teve início mais tardiamente, mas precedendo um período muito sensível do desenvolvimento, a

eclosão, o que indica que, nesta fase, serão necessários níveis mais elevados de protecção, que serão assegurados por estas proteínas em particular. Assim, estes resultados são importantes para suportar o importante papel dos transportadores ABC e das enzimas de biotransformação na protecção dos peixes contra xenobióticos e reflectem que estes grupos de proteínas actuam em cooperação para prover os organismos com um mecanismo efectivo de destoxificação celular.

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Acronyms list

3MC	3-methylcholantrene
4MU	4-methylumbelliferone
7-ER	7-ethoxyresorufin
aa	aminoacid
ABC	Adenosine tris-phosphate Binding Cassette
ABCP	Placenta specific ABC protein
afu	arbitrary fluorescence units
AhR	Aryl-hydrocarbon Receptor
ANOVA	Analysis of variance
Arnt	Aryl-hydrocarbon Receptor translocator
ATP	Adenosine Tris-Phosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BaP	Benzo(a)pyrene
BCRP	Breast Cancer Resistance Associated Protein
BSA	Bovine Serum Albumine
BSEP	Bile Salt Export Pump
BWSF	Biodegradated water-soluble fraction
CAR	Constitutive Androstane Receptor
cDNA	Complementar Deoxyribonucleic Acid
CDNB	1-chloro-2,4-dinitrobenzene
CF	Condition Factor
CIIMAR	Centro Interdiciplinar de Investigação Marinha e Ambiental
CYP	Cytochrome P-450
CYP1A	Cytochrome P450 1A
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4´6-diamino-2-phenylindole
DGV	Direccção Geral de Veterinária

DIC	Differential Interference Contrast
DNA	Deoxyribonucleic acid
DNTPs	Deoxyribonucleotide
dpf	days post-fertilization
EDTA	Ethylenediamine tetraacetic acid
EF1	Elongation factor 1
EPA	United States Environmental Protection Agency
ER	Endoplasmatic reticulum
EROD	Ethoxyresorufin-O-deethylase
EST	Expressed Sequenced Tags
FACs	Fluorescent Aromatic Compounds
FAO	Food and Agriculture Organization
FCT	Fundação para a Ciência e Tecnologia
FD	Fluorescence detector
FF	Fixed wavelength fluorescence
FXR	Farnesoid X Receptor
GA	Glucuronic acid
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GSH	Glutathione
GSTs	Glutathione- S-Transferases
GSTα	Glutathione-*S*-Transferase α
hpf	hours post-fertilization
HPLC	High-performance liquid chromatography
HRP	Horseradish Peroxidase
HSI	Hepatic Somatic Index
HSP90	Heat Shock Protein 90
IHC	Immunohistochemistry
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LOD	Limit of detection

LOQ	Limiti of quantification
LXR	Liver X Receptor
mAb	Monoclonal Antibody
MDR	Multidrug Resistance
MFO	Mixed-Function Oxidase
miRNAs	Micro ribonucleic acids
MO	Monooxygenase enzymes
mRNA	Messenger Ribonucleic Acid
MRPs	Multiresistance Associated Proteins
MSDs	Membrane Spawning Domains
MW	Molecular Weight
MXR	Multixenobiotic Resistance
MZT	Maternal to zygote transition
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
NBDs	Nucleotide Binding Domains
NCBI	National Centre for Biotechnology Information
PAHs	Polycyclic Aromatic Hidrocarbons
PBS	Phosphate Buffer Saline
PC	Pyruvate Carboxylase
PCBs	Polychlorinated Biphenyls
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PFIC	Progressive Familiar Intrahepatic Cholestasis
Pgp	Permeability glycoprotein
PMSF	Phenylmethanesulfonylfluoride
POPs	Persistent Organic Pollutants
PPAR	Proliferator activated Receptor
PPCPs	Pharmaceuticals and Personal Care Products
PTFE	Polytetrafluoroethylene

PXR	Pregnane X Receptor
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribossomal ribonucleic acid
RXR	Retinoid X Receptor
SDS	Sodium Dodecyl Sulphate
SDS-Page	Sodium Dodecyl Sulphate – Polyacrilamide Gel
Spgp	Sister of permeability glycoprotein
TAE	Tris-acetate-ethylenediamine tetraacetic acid
TBS	Tris Buffer Saline
TM	Transmembrane
TMH	Transmembrane Helice
UASEs	Ultrasound AssistedSolvent Extractions
UGTs	Uridine diphosphate glucuronyl transferases
UTAD	Universidade de Trás-os-Montes e Alto Douro
UV	Ultraviolet
WB	Western Blot
XRE	Xenobiotic Responsive Elements
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

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Chapter 1

General Introduction

1. General Introduction

The aquatic ecosystems have unpaired importance for the maintenance and support of the overall environmental health, due to the enormous economic and aesthetic value of aquatic biodiversity. Humans have long depended on aquatic resources for food, medicines, materials, as well for recreational and commercial purposes, such as fishing and tourism. As a result declining levels of aquatic biodiversity, in both freshwater and marine environments, are recognized. Several factors, as species overexploitation, pollution from urban, industrial and agricultural areas, as well as habitat loss and alteration have been contributing to the situation of aquatic environment "health" decline. Although aquatic pollution has a long history, significant international laws to counter it were only enacted in the twentieth century. The aquatic environments were for many years, and still are, the ultimate destination for different type of pollutants from anthropogenic origins. Toxic heavy metals and nanomaterials resultant from industrial processes, persistent organic pollutants (POPs) from agricultural and industrial sources and polycyclic aromatic hydrocarbons (PAHs) mainly resultant from oil spills are among the sources of major concern regarding water pollution, due to their known toxic effects on the aquatic biota. More recently, a highly diverse group of bioactive chemicals, denominated pharmaceuticals and personal care products (PPCPs), have been receiving attention, since these compounds are continually introduced into the aquatic environment as complex mixtures via a number of routes, but primarily by both untreated and treated sewage (Daughton and Ternes, 1999). In order to survive in polluted environments, living organisms have well developed strategies of protection to the adverse effects of pollutants. Whenever possible, organisms avoid or escape from the polluted areas; additionally, chronically exposed organisms use detoxification mechanisms in order to reduce the toxic effects of pollutants. These mechanisms include the activity of specific proteins to preclude the permanence of toxic compounds or their metabolites in the cells - some members of the ATP-Binding Cassette (ABC) superfamily - and enzymatic systems to transform the chemicals into a more easily excreted form - phase I and phase II biotransformation enzymes. Thus, the knowledge of the functionality of these detoxification pathways is of critical importance, to maintain the balance of nature and support the availability of the aquatic resources for future generations.

The main focuses of this PhD thesis are the detoxification mechanisms adopted by fish species, namely the ecotoxicologically relevant ABC efflux transporters and

biotransformation enzymes of phase I and phase II. Recent studies have raised the possibility of a coordinated action between these three groups of proteins, resulting in a powerful and effective mechanism of cellular detoxification, and the cooperation of these systems will be the main focus of this dissertation. To achieve this goal, we have developed *in vivo* assays of exposure of the freshwater fish Nile tilapia (*Oreochromis niloticus*) to Benzo(a)pyrene (BaP), a toxic polycyclic aromatic hydrocarbon (PAH) that is a ubiquitous contaminant of the aquatic environments, and whose carcinogenic and mutagenic effects to the aquatic biota are well recognized. The presence and functionality of ABC transporters and biotransformation enzymes was subsequently evaluated in Nile tilapia barrier tissues (liver, gills and intestine) at gene, protein and activity levels, in order to assess the cooperation between these groups of proteins in the detoxification of this pollutant.

1.1. The superfamily of ABC efflux transporters

The ABC genes represent the largest family of transmembrane proteins encoded in the human genome. These proteins bind to ATP and use that energy to drive the transport of a wide variety of molecules across cellular membranes (table 1.1) (Higgins, 1992; Dean et al., 2001). So far, 56 members of the ABC family have been described, including 48 human ABC genes and 9 additional genes found in other animal species. From the 56 genes, 68% are present in all vertebrate genomes, suggesting that theirs proteins´ structures and functions have been largely conserved throughout the evolution of vertebrate species (Dean and Annilo, 2005). Based on the sequence and the organization of the ATP-binding domains, also known as nucleotide binding domains (NBDs), ABC proteins were grouped into eight subfamilies in eukaryotes (A–H), with seven of these (A–G) present in the human genome (Dean and Annilo, 2005), as shown in table 1.1. The NBDs are located in the cytoplasm and transfer the necessary energy to transport the substrate across the cellular membrane. These domains contain characteristic motifs with specific amino acid sequences (Walker A, Walker B and C-motif) described in all ABC proteins (Walker et al., 1982; Hyde et al., 1990) (figure 1.1b).

Table 1.1– List of known ABC genes, functions and number of members found in human and zebrafish genomes

Subfamily	Members	Functions	Human	Zebrafish*
ABCA	*ABCA1 to ABCA13*	Cholesterol efflux, phosphatidil choline efflux, N-retinylidiene-PE efflux	12 members	7 members
ABCB	*ABCB1 to ABCB11*	Peptide transport; iron transport; Fe/S cluster transport; bile salt transport; xenobiotics transport	11 members	9 members
ABCC	*ABCC1 to ABCC13*	Organic anion efflux, nucleoside transport, chloride ion channel, sulfonylurea receptor, potassium channel regulation, xenobiotics transport	13 members	11 members
ABCD	*ABCD1 to ABCD4*	Very long chain fatty acids transport regulation	4 members	4 members
ABCE	*ABCE1*	Elongation factor complex	1 member	1 member
ABCF	*ABCF1 to ABCF3*	Unknown function	3 members	3 members
ABCG	*ABCG1 to ABCG5*	Cholesterol transport, sterol transport, toxin transport	5 members	5 members
ABCH	*ABCH1*	Unknown function	no members	1 member

* This genome is incompletely assembled and annotated and the gene numbers may be higher. Adapted from Dean and Annilo 2005

Typically, a functional protein contains two NBDs and two membrane spawning domains (MSDs), the latter being composed by 6-10 membrane spawning α-helices that confer the substrate specificity (figure 1.1a) (Locher 2009). Eukaryotic ABC proteins are organized either as full transporters (containing two NBDs and two MSDs), or as half transporters (containing one NBD and one MSD), that have to form homo- or heterodimers in order to constitute a functional protein (Dean et al., 2001). Some variation exists in protein structure in the different subfamilies, as it will be described further ahead, but a high degree of structural and sequence homology is shared among all ABC transporter proteins (Higgins, 2007). The linker region is a central sequence that connects the two homologous halves of the protein, and plays an important role due to its flexible secondary structure that coordinates the functioning of both halves, which is required for the proper interaction of the two ATP binding sites. Evidence exist that, for the efficient ATP hydrolysis, the two NBDs have to interact and form a sandwich dimer, so that C-motif of one NBD comes in contact with Walker-A of the other NBD, forming a nucleotide-binding pocket (Lee et al., 2008).

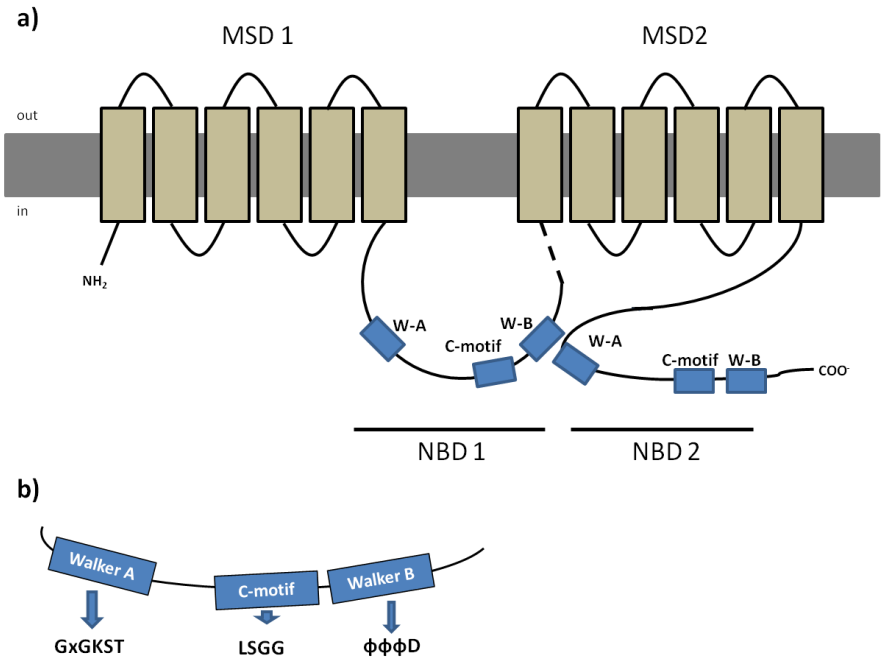

Figure 1.1 – Schematic representation of a typical ABC protein and characteristic amino acid sequences found in Nucleotide Binding Domains (NBDs)

a) lipidic bilayer is shown in grey, membrane spawning domains (MSDs) in light brown and nucleotide binding domains (NBDs) in blue (W-A - Walker A and W-B - Walker B), dashed line represents the linker region; b) aminoacid (aa) sequences characteristic from the conserved domains Walker A (x represents any aa), C-motif and Walker-B (ϕ represents hydrophobic residues). Adapted from Dean et al. 2001.

1.2. Multidrug resistance (MDR) associated ABC transporters

Resistance to multiple anticancer agents is a major impediment for the successful treatment of many forms of malignant tumours. Juliano and Ling (1976) described a multidrug resistance (MDR) phenomenon, found in tumor cell lines of mammals, as the result of low intracellular accumulation of anti-cancer drugs, and related it with the overexpression of a transmembranar protein, responsible for an ATP-dependent efflux of those drugs into the extracellular medium. This protein denominated as Permeability glycoprotein (Pgp) and encoded by the *ABCB1* gene was, for that reason, the first ABC transporter to gain importance and later related to cellular detoxification. From this point, several other members of the ABC transporter efflux family have been identified as capable of transporting endo- or xenobiotic compounds, including members of the ABCC and ABCG subfamilies (Cole et al., 1992; Doyle et al., 1998). Due to their ecotoxicological role, these three subfamilies of transporters will be addressed in higher detail in the following sections.

1.2.1. Subfamily ABCB

1.2.1.1. ABCB1 – P-glycoprotein (Pgp)

The best characterized ABC transporter is Pgp (subfamily B, member 1: ABCB1; MDR1). In humans, Pgp is encoded by two different isoforms of the gene, *MDR1* and *MDR3*, and the class 1 has been implicated in drug resistance whereas the function of class III isoform is still unknown (Georges et al., 1990). ABCB1 (MDR1) was the first eukaryotic ABC member identified in result of its implication in MDR of cancer cells to chemotherapy (Gottesman and Ling, 2006). Further evidence of its MDR abilities included decreased drug accumulation in cells transfected with *ABCB1* gene (Ueda et al., 1987), and increased drug accumulation in gene knockouts organisms, compared to the wild-type organism (Schinkel et al., 1994). In humans, ABCB1 is a 170 - 180 kDa protein containing ~1280 amino acids, with a predicted four-domain structure, typical of most eukaryotic ABC transporters, with two NBDs each preceded by a MSD composed of six transmembrane (TM) helices (Loo and Clarke, 1999) (figure 1.2a). MDR provided by Pgp is a consequence of its remarkable non-specificity with respect to the substrates. Several researchers have focused their attention on the understanding of the promiscuity of this transporter regarding its substrates, and

have indicated common characteristics among them, such as moderate hydrophobicity, small size and positively charged or neutral domains, and include natural products, chemotherapeutic drugs or steroids (reviewed in Schinkel and Jonker, 2003). Pgp can also interact with modulators that are able to reverse MDR by blocking or saturating Pgp binding locations, called chemosensitizers (described in section 1.3).

Besides being expressed in cancerous tissues *ABCB1* is also expressed in normal tissues, such as kidney, intestine, lung, brain, placenta, adrenal cortex, testis, uterus, lymphocytes and hematopoietic cells of mammals, in most cases adopting an apical localization in the cells (reviewed in Szakács et al., 2008). Due to this cellular localization determined in mammals, it is believed that ABCB1 functions in three main areas: 1) limiting the drug entry into the body after oral drug or toxin administration, due to its expression in the apical membrane of enterocytes; 2) once the xenobiotic has reached the blood circulation, ABCB1 promotes drug elimination into bile and urine, as a result of its expression in the canalicular membrane of hepatocytes and luminal membrane of proximal tubule cells in the kidneys, respectively; and, 3) once a xenobiotic has reached the systemic blood-circulation, Pgp limits drug penetration into sensitive tissues, like the blood-brain-barrier (BBB) (Fromm, 2004) where its localized in both luminal and abluminal membranes of capillary endothelial cells, pericytes and astrocytes (Bendayan et al., 2006). These different localizations of ABCB1 in barrier tissues strongly support the role of this transporter as a key player in the cellular defence mechanisms, emphasizing its ecotoxicological relevance.

Besides mammals, highly conserved MDR genes have been described in animals from diverse taxa including nematode worms (Broeks et al., 1995), fruit fly (Vaché et al., 2006), plants (Wang et al., 1996) and bacteria (van Veen et al., 1998).

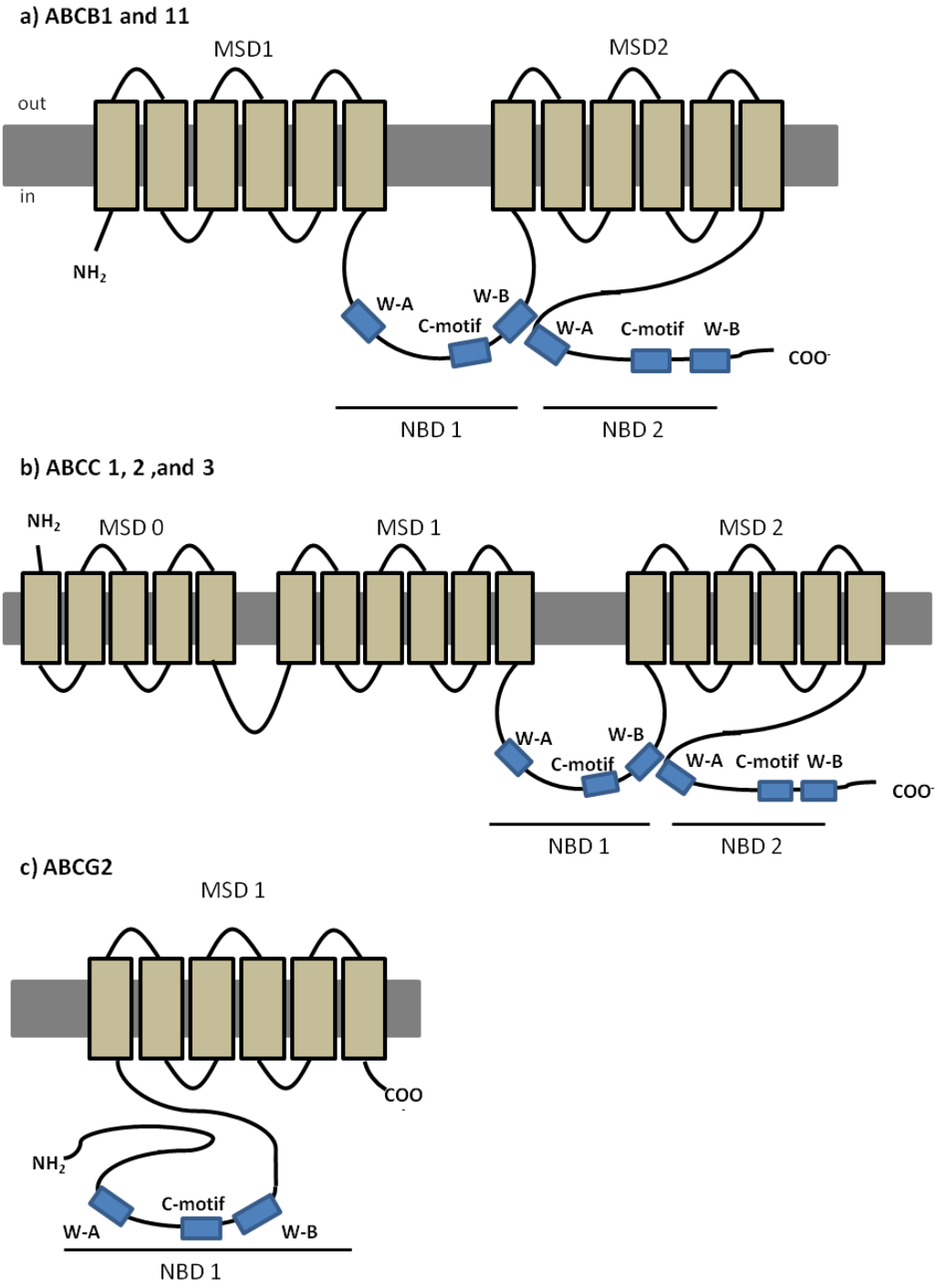

Figure 1.2 – Predicted structures of MDR associated members of ABC transporters

a) predicted structure of ABCB1 and ABCB11; b) predicted structure of "long-chain" ABCC proteins, ABCC1, 2 and 3; c) predicted structure of ABCG2.

1.2.1.2. ABCB11 – Spgp or BSEP

Sister of P-glycoprotein (Spgp, ABCB11, BSEP) was first cloned from porcine liver (Childs et al., 1995), and named for its close sequence homology with Pgp. It follows the same predicted structure as ABCB1 (figure 1.2a). Recently, this transporter has become more widely known as the bile salt export pump (BSEP), since studies in rats showed predominant expression in liver, canalicular localization, and high affinity to transport primary and secondary bile salts (Gerloff et al., 1998). ABCB11 is thus positioned to mediate active, ATP dependent, bile acid secretion across the canalicular membrane of hepatocytes, limiting intracellular bile acid concentrations and maintaining the enterohepatic circulation of bile acids. Conclusive evidence that BSEP is indeed the bile salt export pump in liver came from a study in which mutations in the human ABCB11 gene resulted in type 2 progressive familial intrahepatic cholestasis (PFIC2), a disease characterized by an interruption in hepatic bile salt secretion (Strautnieks et al., 1998). Despite its predominance in liver, ABCB11 was also found in other porcine tissues, such as large and small intestinal mucosa and in brain (Török et al., 1999). This suggests that ABCB11 may perform additional, extrahepatic functions, possibly limiting other tissues exposure to bile salts. As well as its endogenous role as a hepatic bile salt export pump, studies in mammals cell lines transfected with murine *ABCB11* showed that ABCB11 was capable of transporting some Pgp substrates (vinblastine and calcein-acetoxymethyl ester), although it was unable to efflux others (rhodamine 123, vincristine, daunorubicin, paclitaxel, and digoxin) (Lecureur et al., 2000). Thus the role of ABCB11 in drug disposition, if any, is considerably more limited than that of its close relative, Pgp.

1.2.2. Subfamily ABCC (MRPs – Multiresistance associated proteins)

After the discovery of ABCB1, the study on cancer cells displaying MDR phenotype not associated with ABCB1 expression, led to the discovery of ABCC1, the founding member of the ABCC subfamily (Cole et al., 1992). So far, this subfamily includes a total of 13 members, most of which are active ATP-dependent membrane transporters for organic anions of therapeutic compounds (Honorat et al., 2009). Among its members, at least five (ABCC1, ABCC2, ABCC3, ABCC4 and ABC5) are potentially involved in mediating drug resistance (Cole et al., 1992; Kool et al., 1997; Evers et al., 1998). At present, there are no high-resolution structural data available on any eukaryotic ABCC transporter, but considering predicted membrane topologies, phylogenetic position and domain

arrangements, members of the ABCC subfamily can fall in one of two different subclasses, “short” and “long” (Kast and Gros, 1997; Bakos and Homolya, 2007; Honorat et al., 2009). The so called “long” ABCC transporters (ABCC1, 2, 3 and 6) present an additional N-terminal MSD (MSD0), of approximately 250 amino acids, a unique feature of these specific transporters (figure 1.2b) in comparison to the “short” transporters. ABCCs are ~190kDa proteins, and share only 14-25% amino acid identity with ABCB proteins (Cole et al., 1992; Keppler and Konig, 1997). From the ABCC subfamily, ABCC1 and ABCC2 are the best characterized transporters with existing evidence from animal models to have a role in tissue defence, while other members like ABCC3, 4 and 5, are far less studied and characterized. The toxicological relevance of ABCC1 and ABCC2 will be evaluated in this dissertation. *ABCC2* gene was identified based on its similarity to *ABCC1* and absence of its expression of homozygously *ABCC2*-deficient rats and humans (Mayer et al., 1995; Keppler and KÖNig, 2000). The absence of the *ABCC2* gene in hepatocytes canalicular membrane causes the Dubin–Johnson syndrome (Konig et al., 1999), and affected individuals suffer from a conjugated hyperbilirubinemia, since ABCC2 normally mediates the hepatobiliary excretion of bilirubin (König et al., 1999). As for ABCB1, ABCC1 and ABCC2 are also expressed in non-malignant tissues. In mammals, ABCC1 shows a ubiquitous expression throughout the different tissues, with relatively high levels found in kidney, lung, testis, skeletal muscle, peripheral blood mononuclear cells, while relatively low levels were found in liver (Cole et al., 1992; Flens et al., 1996; Stride et al., 1996). Similarly, ABCC2 has also been found in tissues important for the pharmacokinetics of substrate drugs, namely liver, kidney, placenta, lungs, intestine and BBB (for a review see Schinkel and Jonker, 2003). Although without a complete overlap, many similarities exist between the spectrum of compounds transported by ABCC1 and ABCC2, which are mainly composed by amphipathic anionic drugs and endogenous compounds, encompassing GSH-, glucoronide- and sulphate- conjugates (reviewed in Schinkel and Jonker, 2003). In most tissues, ABCC1 efflux transporter is localized in the basolateral surface of the cells, which, in certain tissues, results in the efflux of its substrates into the blood (Evers et al., 1996). In contrast to ABCC1, and similarly to ABCB1 and ABCB11, ABCC2 is localized in the apical membrane of the cells in which it is expressed (Evers et al., 1998).

1.2.3. Subfamily ABCG – ABCG2, BCRP, MXR or ABCP

The second member of the ABCG subfamily, ABCG2, is a ~72kDa efflux transporter, whose overexpression in permanent cell-lines has been associated with high levels of resistance to a variety of anticancer drugs, such as mitoxantrone, doxorubicin, and daunorubicin, without evidence of expression of the well-characterized genes for *ABCB1* or *ABCC*s therefore contributing to a MDR phenotype. Moreover reduced accumulation of such drugs could be reversed by incubation of the cells in ATP-depleting conditions, indicating the presence of an ATP-dependent transporter (Ross et al., 1999; Bates et al., 2001; Allen and Schinkel, 2002). This protein is also known as breast resistance associated protein (BCRP) (Doyle et al., 1998), mitoxantrone-resistance protein (MXR) (Miyake et al., 1999) or placenta-specific ABC protein (ABCP) (Allikmets et al., 1998) since it was cloned independently by 3 different groups. Members of the ABCG subfamily have a unique domain organization; unlike the remaining subfamilies, these are half-transporters, composed by one single NBD followed by one MSD (figure 1.2c). In addition, they also present a unique protein configuration, in which the NBD precedes the MSD, whereas ABCBs and ABCCs have an opposite domain arrangement, that is, the MSD is followed by the NBD (figure 1.2c). There are increasing evidence to suggest that these proteins may operate either as homodimers or heterodimers (Graf et al., 2003; Xu et al., 2004). ABCG2 is believed to function probably as a homodimer (Doyle et al., 1998; Miyake et al., 1999; Kage et al., 2002) or homotetramer (Xu et al., 2004).

Functional characterization studies have demonstrated that ABCG2 can transport a wide range of substrates, from chemotherapeutic agents to organic anion conjugates (reviewed in Mao, 2005). Moreover, it seems that ABCG2 has higher affinity to transport sulphated conjugates of steroids and xenobiotics over GSH and glucoronide metabolites (Chen et al., 2003). High levels of ABCG2 have been found in placenta, brain, prostate, intestine, liver and ovary, mostly in an apical localization (Rocchi et al., 2000; Scheffer et al., 2000; Maliepaard et al., 2001).This tissue localization is consistent with the ability of ABCG2 to function as a protective efflux pump, limiting absorption of drugs and increasing elimination of its substrates.

1.3. From MDR to MXR - ABC efflux transporters in aquatic organisms

Aquatic organisms are able to survive and thrive in heavily polluted environments, showing surprisingly low accumulation of pollutants in body tissues (Kurelec and Pivčević, 1989). Kurelec and coworkers were the first to demonstrate that aquatic organisms adopt strategies of xenobiotic transport, in order to improve adaptation to pollutants in their habitats. To this phenomenon of resistance, Kurelec coined the term of multixenobiotic resistance (MXR) (Kurelec, 1992), and identified its biochemical basis as similar to the one adjacent to the MDR phenotype. The presence of a drug transporter resembling Pgp (ABCB1) sensitive to verapamil and trypsin (known inhibitors of human Pgp) was described in two bivalves species (*Anodonta cygnea* and *Mytilus galloprovinciallis*) (Kurelec and Pivčević, 1989, 1991). This study was the starting point for the identification and characterization of the MXR phenotype in several other species of aquatic organisms, such as sponges (Kurelec et al., 1992), molluscs (McFadzen et al., 2000; Minier et al., 2002; Smital et al., 2003; Luckenbach and Epel, 2008; Faria et al., 2011), crabs (Kohler et al., 1998) and sea urchins (Toomey and Epel, 1993; Hamdoun et al., 2002). In fish, the presence of ABC efflux transporters have been described for an increasing number of species, such as winter flounder (Chan et al., 1992), rock cod (Zucchi et al., 2010), rainbow trout (Zaja et al., 2008; Fischer et al., 2010; Loncar et al., 2010), zebrafish (Long et al., 2011a; Long et al., 2011b), mullet (Diaz de Cerio et al., 2012) and killifish (Paetzold et al., 2009) (table 1.2).

Identification of ABC transporters and characterization of the MXR mechanism has been achieved by the use of several detection methods, including quantitative or semi-quantitative reverse transcription polymerase chain reaction (qRT-PCR or RT-PCR) and *in situ* hybridization to evaluate mRNA expression of genes, immunochemical techniques for protein detection applying mammals monoclonal antibodies to ABCB1 (western blotting or immunohistochemistry), northern-blotting and activity assays (through the measurement of efflux or accumulation of fluorescent model substrates).

Most studies aiming to determine tissue distribution pattern of ABC transporters in aquatic organisms have been directed to Pgp by the use of the mammalian anti-Pgp monoclonal antibody (mAb) C219. This mAb recognizes an epitope common to all known Pgps (in human MDR1 and MDR3) and also to Spgp (ABCB11) (Georges et al., 1990; Childs et al., 1995; van den Elsen et al., 1999). After probing with the mammalian mAb C219, an immunologically related to the mammalian MDR protein was identified in embryos and gills of a few aquatic invertebrates (Toomey and Epel, 1993; Cornwall et al., 1995). In

fish, a positive reaction was seen in the hepatic bile canaliculi (Kleinow et al., 2000; Doi et al., 2001; Bard et al., 2002a; Bard et al., 2002b; Klobučar et al., 2010), apical membrane of enterocytes (Kleinow et al., 2000; Doi et al., 2001; Bard et al., 2002a), kidney (Kleinow et al., 2000) and in endothelial capillaries of brain (Miller et al., 2002). A negative reaction of the mAb C219 was patent in gills, kidney, gonad, brain, spleen, heart of killifish and blenny (Bard et al., 2002a; Bard et al., 2002b). In summary, the suggested Pgp localization in fish reveals a pattern similar to that described in mammals, with Pgp present in epithelial tissues involved in secretion, absorption or serving a barrier function. However, due to the known cross-reactivity of the mAb C219 with ABCB11 (Childs et al., 1995), results from these studies should be addressed with care, since until the development of fish-specific Pgp probes, ABCB1 definitive tissue localization cannot be demonstrated in fish (Sturm and Segner 2005).

For other ABC efflux transporters no fish-functional antibodies are available, thus information on their distribution pattern is quite limited and restricted to the mRNA expression among the various fish tissues. As in mammals, *ABCB11* was found to be almost exclusively expressed in trout liver, with low expression in proximal intestine, and very low expression in other tissues as gills, brain, gonads and kidney (Loncar et al., 2010), supporting its role in the efflux of bile salts from hepatocytes into the bile. In trout tissues, low *ABCC1* expression was found, and kidney was the only tissue showing notable *ABCC1* expression while significant expression levels of *ABCC2* in major detoxification tissues were reported (Loncar et al., 2010). High *ABCC2* expression was also previously reported in kidney, intestine and liver of little skate (Cai et al. 2003) and in apical membrane of proximal kidney tubule of killifish (Miller et al., 2007). Regarding ABCG2, Loncar and coworkers (2010) have examined the tissue distribution pattern of this protein in trout tissues, and found high expression levels in gonads and moderate expression in distal part of intestine, kidney and brain. Relatively high expression of *ABCG2* was reported in trout liver and primary hepatocytes (Zaja et al., 2008) (table 1.2).

Based on these results and on mammal studies, investigators believe that, in aquatic organisms, ABC eflux transporters should follow a similar distribution pattern and cellular localization, with expression in tissues involved in secretion, absorption or serving a barrier function, like liver and intestine (figure 1.3). Thus, ABCB1, ABCB11, ABCC2 and ABCG2 should be localized in the apical membrane of polarized cells, pumping substrates into the intestinal lumen or bile canaliculi, while ABCC1 localization in basolateral membrane results in the export of its substrates into the blood. Additionally, ABCB1 should efflux mostly unmodified compounds, while ABCCs and ABCG2 should efflux organic anions conjugated by phase II enzymes (figure 1.3) (reviewed in Epel et al., 2008).

Table 1.2 – ABC transporters involved the efflux of toxic compounds, fish species where they were identified and tissue distribution pattern in mammals and fish.

	Mammals		Fish			
Protein	**Tissue distribution**	**Localization in polarized cell**	**Specie**	**Common name**	**GenBank accession number**	**Tissue distribution**
ABCB1	BBB, liver, intestine, kidney, placenta, stem cells	apical	*Barbus barbus*	Barbel	DQ059069.1	liver, intestine, kidney, brain, gonads[1,2,3,4,6,7]
			Carassius auratus	Goldfish	DQ059072.1	
			Chondrostoma nasus	Nase	AY948951.1	
			Cyprinus carpus	Carp	AY999964.1	
			Danio rerio	Zebrafish	XP_001922717	
			Dicenthrarchus labrax	European seabass	GQ273979.1	
			Fundulus heteroclitus	Killifish	AF099732.1	
			Leuciscus cephalus	Chub	AY999966	
			Mullus barbatus	Red mullet	AY850375.1	
			Oncorhynchus mykiss	Rainbow trout	AY863423.3	
			Platichthys flesus	European flounder	AF175686.1	
			Pleuronectes americanus	Winter flounder	AY053461.1	
			Poeciliopsis lucida	Topminnow	DQ842514.2	
			Scophtalmus maximus	Turbot	AJ291813	
			Trematomus bernacchii	Rock cod	FJ938210.1	
ABCB11	liver	apical	*Danio rerio*	Zebrafish	XP_001923538	liver, intestine[1]
			Dicenthrarchus labrax	European seabass	GQ273980.1	
			Fundulus heteroclitus	Killifish	AF135793.1	
			Oncorhynchus mykiss	Rainbow trout	DQ865266.2	
			Platichthys flesus	European flounder	AJ344042.1	
ABCC1	lung, testis, kidney, peripheral blood mononuclear cells, skeletal and cardiac muscle, placenta	basolateral (apical in brain endothelial cells)	*Barbus barbus*	Barbel	FJ890350.1	testis, ovary, kidney, muscle, gills, heart, liver, intestine, brain, eye[1,8]
			Danio rerio	Zebrafish	XM_002661202	
			Dicenthrarchus labrax	European seabass	GQ273982.1	
			Oncorhynchus mykiss	Rainbow trout	GQ166973.1	
			Oryzias melastigma	Medaka	JN629038.1	
			Platichthys flesus	European flounder	AJ344044.1	

Table 1.2 (continued)

			Poeciliopsis lucida	Topminnow	HM102361.1	
			Trematomus bernacchii	Rock cod	FJ938212.1	
ABCC2	BBB, liver, intestine, kidney, placenta, lung	apical	*Carassius auratus*	Goldfish	FJ890349.1	liver, kidney, intestine, brain, muscle[1,9,10,11]
			Chondrostoma nasus	Nase	AY948950	
			Cyprinus carpio	Carp	AY679169	
			Danio rerio	Zebrafish	NM_200589.1	
			Dicenthrarchus labrax	European seabass	GQ273983.1	
			Leuciscus cephalus	Chub	FJ890348.1	
			Mullus barbus	Barbel	AY275434.1	
			Oncorhynchus mykiss	Rainbow trout	NM_001124655.1	
			Plactichthys flesus	European flounder	AJ344045.1	
			Poeciliopsis lucida	Topminnow	HM102360.1	
			Trematomus bernacchii	Rock cod	FJ938211.1	
			Raja erinácea	Little skate	AF486830	
ABCG2	BBB, placenta, liver, intestine, breast, stem cells	apical	*Chelon labrosus*	Mullet	HM467811.1	liver, kidney, gonads, intestine, gills[1]
			Dicenthrarchus labrax	European seabass	GQ273981.1	
			Oncorhynchus mykiss	Rainbow trout	EU163724.1	
			Poeciliopsis lucida	Topminnow	HM102358.1	
			Salmo salar	Atlantic salmon	NM_011736655.1	

1(Loncar et al., 2010), 2 (Kleinow et al., 2000), 3(Doi et al., 2001), 4(Bard et al., 2002a), 5(Bard et al., 2002b), 6(Klobučar et al., 2010), 7(Miller et al., 2002), 8(Long et al., 2011a), 9(Long et al., 2011b), 10(Miller et al., 2007), 11 (Cai et al., 2003)

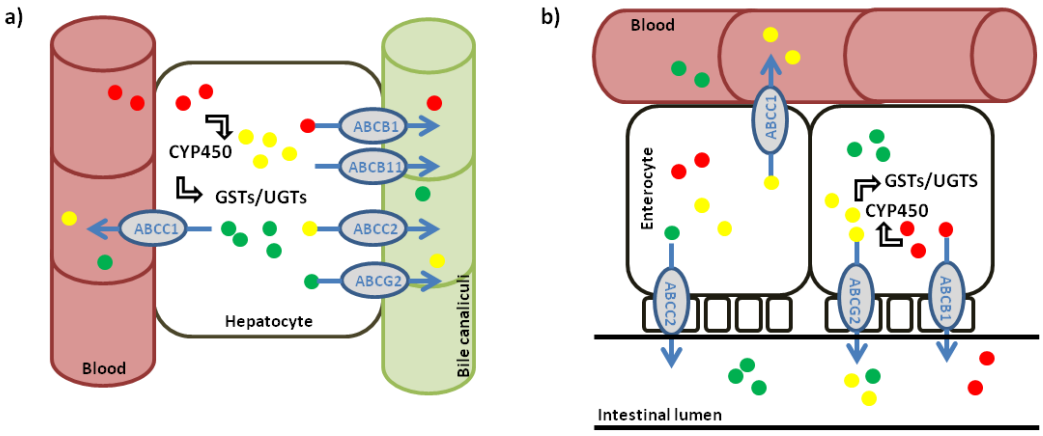

Figure 1.3 - Schematic representation showing probable localization of ABC transporters in liver and intestine of fish, as determined in mammals.

(a) liver hepatocytes, b) enterocytes. Parent xenobiotic compounds, phase I metabolites and phase II metabolites are shown as red, yellow and green circles, respectively. Adapted from Schinkel and Jonker 2003, Brand et al., 2006.

The bulk of the studies conducted in aquatic organisms, on the interaction of environmental contaminants with ABC efflux transporters have been mostly performed on Pgp. Some compounds, as Pgp model reversal agents (verapamil and/or cyclosporine A) and xenobiotics (like the insecticide malathion) were shown to inhibit Pgp activity in fishes, resulting in an increase of the bioaccumulation of toxic compounds like 2-aminoanthracene, hydrocarbon-rich Diesel-2 oil, and/or model Pgp substrates like rhodamine B (Kurelec et al., 1992; Kurelec, 1995; Smital and Sauerborn, 2002). Other studies have demonstrated the ability of some xenobiotics to induce Pgp expression in fish, such as cadmium (Zucchi et al., 2010), the organophosphate insecticide chloropyrifos and the carcinogen N-nitrosodiethylamine (Albertus and Laine, 2001). Similarly, resistance-killifish populations from highly polluted sites also demonstrated enhanced Pgp expression (Cooper et al., 1999; Bard et al., 2002a) suggesting a protective role of Pgp. Nevertheless, in another study performed in killifish from a heavily polluted site with polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and heavy metals, Paetzold and coworkers (2009) found no up-regulation of hepatic *ABCB1* transcripts. Moreover, after *in vivo* exposures of fishes to different xenobiotics, such as prochloraz, nonylphenol diethoxylate, ß-naphthoflavone, BaP and 3,4,3´,4´-tetrachlorobiphenyl no changes were seen in the levels of hepatic and/or intestinal Pgp (Doi et al., 2001; Sturm et al., 2001).

Few studies have addressed the response of other ABC efflux transporters upon animals' exposure to pollutants. Paetzold and co-workers (2009) described an up-regulation of hepatic transcripts for *ABCC2* and *ABCG2* in a multiresistant population of killifish living in a pond heavily polluted with PAHs, PCBs and heavy metals, while no up-regulation was seen in *ABCB1* and *ABCB11* mRNA. Expression of *ABCC2* in excretory organs of zebrafish including kidney, liver and intestine was induced by exposure to heavy metals (Long et al., 2011b). Moreover, an up-regulation of *ABCC2* was observed in red mullet liver from an oil contaminated site (Della Torre et al., 2010). In another study, Long and co-workers have described an up-regulation of *ABCC1* gene in ZF4 cells and embryos of zebrafish exposed to heavy metals (Long et al., 2011a; Long et al., 2011b). Similarly, *ABCB1* and *ABCC2* transcripts were also up-regulated in liver of Cd-exposed *Trematomus bernachii* (Zucchi et al., 2010).

These findings demonstrate that, although some compounds have been shown to interact with ABC efflux transporters data remains fragmented and largely restricted to laboratory observations. It remains uncertain which classes of environmental toxicants can induce and/or repress the activity of these proteins, and to what extent this MXR mechanism protects aquatic animals from the toxic action of xenobiotics. Most ABC efflux transporters are remarkably non-specific with respect to its substrates. This feature can be an important advantage, as it provides protection against many novel anthropogenic products, but it can also render the system vulnerable to the presence of multiple substrates that will compete for binding sites, saturating these locations, or directly inhibit the transporter activity (chemosensitization). Thus, there is a fundamental need to identify the common features of ABC efflux transporters substrates, but also of its inhibitors. Chemosensitization, can compromise the effectiveness of the defence system, since toxic substances that would normally be excluded, will, in those situations, remain in the cell and exert their toxic effects (Higgins, 2007). While in clinical research the goal is to inhibit the activity of these efflux proteins, in a toxicological approach their optimal functioning is of critical importance.

Research on these proteins has been delayed due to the inexistence of specific probes/antibodies for these transporters in aquatic organisms. Hence, there is the need to overcome this limitations, and direct further investigations to the MXR mechanism in order to fully understand the physiological and toxicological functions of ABC transporters in aquatic organisms, including fish.

1.4. Biotransformation enzymes

Biotransformation of xenobiotics is a well known and described strategy adopted by living organisms to cope with the presence of xenobiotics present in their habitats. This detoxification mechanism consists on a two-phase process of enzymatic reactions that alter the chemistry of non-polar lipophilic chemicals to polar water soluble metabolites, leading to the detoxification and elimination of the parent compound (Black and Coon, 1987; Buhler and Williams, 1989). Biotransformation alters the toxicity of pollutant compounds, resulting in either a decrease of the toxicity of the compound followed by its excretion, or in the formation of more reactive metabolites, which leads to increased toxicity (for a review see van der Oost et al., 2003). Originally found in mammalian liver, biotransformation enzymes were later described in several other animals, plants, bacteria and fungi (Nelson et al., 1996). In mammals, the activity and or expression of biotransformation enzymes is found in almost all tissues, but due to its function, position and blood supply, liver is considered to be the major organ involved in the biotransformation of xenobiotics in all species (Guengerich, 1993). Nevertheless, other organs that function as the body´s first-pass barrier are often endowed with biotransformation capacity, such as skin, intestine and lung in mammals (Raunio et al., 1995) and, in aquatic organisms, gills and intestine (McElroy and Kleinow, 1992; James, 1997; Jönsson et al., 2004; Jönsson et al., 2006; Nahrgang et al., 2010a; Nahrgang et al., 2010b). Unlike ABC efflux transporters, the characterization of biotransformation enzymes is well known for aquatic organisms, including fish. Thus, throughout the following sections, we will mostly focus on the studies performed with fish species and their major findings regarding drug metabolizing enzymes.

1.4.1. Phase I enzymes

The enzymes of phase I metabolism (oxidation, reduction, hydration, hydrolysis) introduce a functional group (-OH, -COOH, -NO2, etc.) into the xenobiotic, therefore providing a non-synthetic alteration of the parent compound (Commandeur et al., 1995). For the majority of xenobiotics, phase I reactions are catalysed by microsomal monooxygenase (MO) enzymes, also known as the mixed-function oxidase (MFO) system (i.e. cytochrome P450, cytochrome b_5 and NADPH cytochrome reductase). The cytochrome P450s (CYPs) constitute a superfamily of heme-containing proteins that

catalyse biological oxidation and reduction reactions, and are predominantly located in the endoplasmic reticulum (Stegeman et al., 1992). Currently 18 CYP gene families have been identified in fish (CYP1-8, CYP11, CYP17, CYP19-21, CYP24, CYP26-27, CYP39, CYP46 and CYP51) (Nelson, 2003). Members of the CYP family responsible for xenobiotics metabolism belong to the subfamilies CYP1, CYP2, CYP3 and CYP4, and CYP1A is the most studied isoform in fish, and has been frequently used as a biomarker to assess contamination in the aquatic environment (reviewed in Uno et al., 2012). In our study, CYP1A activity and expression was used as indicative of phase I metabolism, thus it will be described in further detail in the following section.

1.4.1.1. CYP1A

cDNAs encoding for CYP1A enzymes have been isolated from several fish species such as rainbow trout (Råbergh et al., 2000), mummichog (Morrison et al., 1998), European seabass (Stien et al., 1998), Atlantic salmon (Arukwe, 2002), medaka, mangrove rivulus (Kim et al., 2004), yellow catfish (Kim et al., 2008) and crucian carp (Fu et al., 2011). As in mammal studies, results from fish studies have shown that the mRNA expression of *CYP1A* is regulated by the aryl hydrocarbon receptor (AhR), and that this cytosolic transcription factor has, in turn, a high affinity to planar compounds, such as dioxins and PAHs (van der Oost et al., 2003).

The mechanism of AhR-mediated induction of CYP1A is initiated by the binding of the ligand (e.g. a xenobiotic) to the citosolic AhR, which exists as a multiprotein complex with heat shock protein 90 (HSP90). Ligand binding causes a conformational change that facilitates nuclear translocation and association with the AhR nuclear translocator (Arnt). In the nucleus the AhR/Arnt heterodimer, associates with xenobiotic responsive elements (XRE) to initiate the transcription and translation of AhR-responsive genes, including CYP1A (Schlenk et al., 2008) (figure 1.4). The evaluation of phase I metabolism can be done with the use of different approaches, like the measurement of the mRNA levels, immunochemical methods to measure CYP1A-reactive protein or by measurement of CYP1A isoenzyme catalytic activity. The measurement of ethoxyresorufin-O-deethylase (EROD) activity in fish is a well-established and highly sensitive biomarker of exposure to PAHs and other structurally similar compounds (Bucheli and Fent 1995; Stegeman and Hahn 1994), providing evidence of receptor-mediated induction of CYP1A by xenobiotic chemicals. EROD activity describes the rate of the CYP1A mediated deethylation of the substrate 7-

ethoxyresorufin (7-ER) to form the product resorufin, being an indication of the amount of CYP1A enzyme present (Whyte et al., 2000).

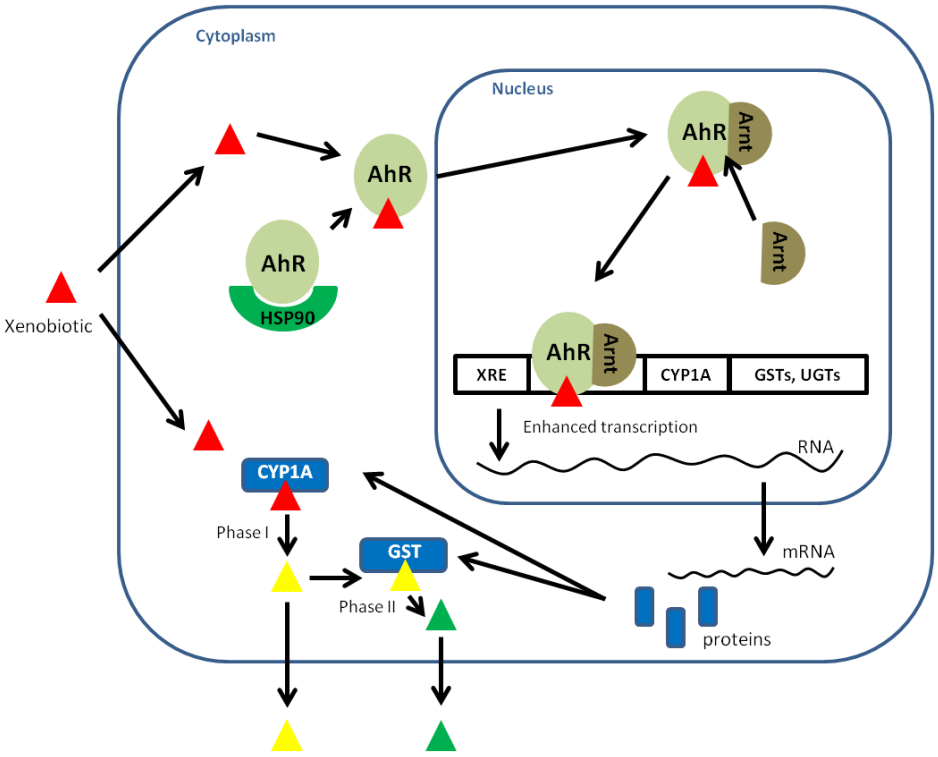

Figure 1.4 - Schematic representation of the mechanisms involved in biotransformation of xenobiotic compounds in cells.

AhR, aryl hydrocarbon receptor; HSP90, heat shock protein 90kDa; Arnt, aryl receptor nuclear translocator; XRE, xenobiotic responsive elements; CYP1A, cytochrome P450 1A; GSTs, glutathione-S-transferases; UGTs, UDP-glucoronyl transferases. Adapted from van der Oost et al., 2003.

Using one or more these evaluation methods, CYP1A presence was reported in liver, and also in various extra-hepatic tissues of aquatic organisms, such as gills, intestine, olfactory epithelium and heart (Smolowitz et al., 1992; Van Veld et al., 1997; Jönsson et al., 2006). Moreover, it has been demonstrated in different fish species that CYP1A expression and/or activity is strongly induced by different xenobiotic compounds, such as PAHs, PCBs and dioxins (James, 1997; Richardson et al., 2001; Ferreira et al., 2006; Calò et al., 2009). All these evidence make phase I metabolism as a vital process, among the mechanisms adopted to deal with the threat of pollutant exposure, and to diminish deleterious effects. In addition, the knowledge on CYP1A regulation makes it a validated biomarker applied in environmental risk assessment.

1.4.2. Phase II enzymes

Phase II of the biotransformation process involves the conjugation of the xenobiotic parent compound or its phase I metabolites with an endogenous ligand. Conjugations are addition reactions in which large and often polar chemical groups or compounds, such as sugars or amino acids, are covalently added to the xenobiotic chemical (Lech and Vodicnik, 1985). The majority of phase II enzymes catalyse these synthetic conjugations, thus facilitating the excretion of chemicals by the addition of more polar groups (e.g. glutathione (GSH), glucuronic acid (GA), sulphate) (Schlenk et al., 2008). Accordingly to their cofactor, examples of phase II enzymes are gluthathione-S-transferases (GSTs), UDP-glucuronyl transferases (UGTs) and sulfotransferases (SULT).

Studies in fish have shown that the activity of some phase II enzymes, like GSTs and UGTs, are under the regulatory pathways of the AhR, and that UGTs genes can also be induced via the nuclear pregnane X receptor (PXR) (George, 1994; Schlenk et al., 2008) (figure 1.4). In our study, GSTs and UGTs expression and/or activity was used as indicative of phase II metabolism, thus these enzymes will be described in higher detail in the following sections.

1.4.2.1. GSTs (Glutathione-S-Transferases)

GSTs are a multigene superfamily of dimeric, multifunctional enzymes, widely distributed throughout all eukaryotic species (Hayes and Pulford, 1995b). The majority of GSTs catalyze the conjugation of GSH with compounds that contain an electrophilic centre, through the formation of a thioether bound between the sulphur atom of GSH and the substrate (Chasseaud, 1979; Mannervik, 1985). Two major superfamilies of mammalian GSTs with transferase activity have been described, cytosolic and membrane-bound (Hayes and Strange, 2000). Based on their sequence homology, cytosolic mammalian enzymes have been assigned into one of eight families or classes, Alpha (α), Mu (μ), Pi (π), Sigma (σ), Theta (θ), Zeta (ζ), Omega (ω) and Kappa (κ) (Mannervik, 1985; Meyer et al., 1991; Pemble et al., 1996; Board et al., 1997; Board et al., 2000). The second group of membrane-bound transferases was more recently discovered and is composed by at least six families (Jacobsson et al., 2000). The soluble, or cytosolic, GSTs appear to be primarily involved in the metabolism of foreign chemicals, such as carcinogens, environmental pollutants, and cancer chemotherapeutic drugs, as well as detoxification of potentially harmful

endogenously derived reactive compounds (Hayes and Pulford, 1995a). Additionally, many endogenous GST substrates are formed as a consequence of macromolecules modification by reactive oxygen species, and these transferases are also considered to have an antioxidant function (Hayes and McLellan, 1999). In addition to the measurement of the mRNA levels for the different GST isoforms, in a great number of studies GST is evaluated by the determination of the total catalytic activity using the artificial substrate 1-chloro-2,4-dinitrobenzene (CDNB), which is conjugated by the majority of GST isoforms (van der Oost et al., 2003). GST activity and/or expression has been frequently used as a biomarker of exposure to xenobiotic compounds in studies conducted with aquatic organisms to evaluate environmental contamination levels (Martínez-Lara et al., 1996; Perez-Lopez et al., 2002; Ferreira et al., 2008; Della Torre et al., 2010; Kim et al., 2010). Nevertheless, the feasibility of this enzyme as a biomarker of exposure is a matter of discussion, since in a high number of studies no significant alterations have been described in the activity of these enzymes after contaminant exposure (van der Oost et al., 2003).

Nevertheless, these enzymes display crucial functions in the detoxification process facilitating the excretion of xenobiotics by increasing their polarity, which can then be easily translocated by ABC transporters due to a high affinity of these proteins to phase II metabolites, mainly ABCCs and ABCG2.

1.4.2.2. UGTs (UDP-glucuronyl transferases)

UGTs, represent another major group of phase II conjugating enzymes in vertebrate species. In addition to their role in the biotransformation of natural toxins and anthropogenic contaminants, UGTs are also active in the metabolism of endogenous compounds, such as steroids and thyroid hormones, and waste products such as bilirubin (Dutton, 1990). Due to their importance in the breakdown of therapeutic drugs, extensive research of these phase II enzymes has been performed in the medical and pharmaceutical fields (Parkinson, 2001). These phase II enzymes are located in the endoplasmatic reticulum (ER), and catalyse the conjugation of GA to a wide variety of acceptor substrates to form ß-glucuronides, increasing the water solubility of the substrates, thus facilitating transport and excretion. In mammals, 17 UGT genes have been characterized, subdivided in two different families (1 and 2). Expressed sequence tags (ESTs) have been cloned from several fish species, and analysis of the currently published zebrafish EST sequences, and the latest assembly of the genome, reveals

that at least 14 distinct UGTs are expressed (Schlenk et al., 2008). UGT isoforms are found in a variety of fish species, and, as in mammalian systems, a diverse group of compounds have been identified as UGTs substrates (e.g. chlorinated phenols, aromatic hydrocarbons metabolites, pesticides) (George, 1994). The liver is quantitatively the most important site for glucuronidation of xenobiotics in fish, nevertheless significant activities have also been detected in extrahepatic tissues, including kidney, gills and intestine (George, 1994).

UGT activity is often used as a biomarker of exposure to xenobiotic compounds in studies conducted with aquatic organisms to evaluate environmental contamination levels, and generally an increase of hepatic UGT activity is reported, either in laboratory or field studies (van der Oost et al., 2003). However, low sensitivity of this enzyme has also been reported in field studies conducted in fish (Schreiber et al., 2006; Della Torre et al., 2010).

1.5. From phase 0 to phase III – a complete pathway for cellular detoxification

Biological membranes are barriers for the uptake, distribution and elimination of xenobiotics in the organism (Simkiss, 1995). Bioaccumulation of organic compounds occurs mainly passively, driven by the compound´s hydrophobicity. Once absorbed, the xenobiotic may undergo a detoxification pathway, in order to be excreted from the organism before it exerts toxic effects. The most studied pathway for cellular detoxification is the biotransformation of xenobiotics by phase I and phase II enzymes. In recent years it has been proposed that two additional steps of drug disposition, called phase 0 and phase III, are equally important as biotransformation (Szakács et al., 2008). These phases involve the modulation of cellular efflux, by ABC transporters, of either unmodified or metabolized compounds. Thus, xenobiotic efflux transporters and biotransformation enzymes may be part of a coordinated defence mechanism that protects cells from xenobiotic insults (Xu et al., 2005). Evidence to this coordination came from mammalian models, where several receptors, including the AhR and orphan nuclear receptors, such as liver X receptor (LXR), pregnane X receptor (PXR), farnesoid X receptor (FXR), peroxissome proliferator activated receptor (PPAR), constitutive androstane receptor (CAR), retinoid X receptor (RXR), were shown to be key mediators of drug-induced changes in phase I and phase II metabolizing enzymes, as well as phase III transporters involved in efflux mechanisms (reviewed in Xu et al.,

2005) (figure 1.5). However, whether phase I and II enzymes inducers coordinately regulate ABC efflux transporters genes requires further studies that can shed some light on the roles of receptors, transcription factors and signalling cascades of this metabolism/transport of endogenous and exogenous compounds (Xu et al., 2005). Moreover, in mammals, 1) induction of Pgp has been reported after exposure to known inducers of phase I (CYP1A) and phase II enzymes, like BaP and 3-methylcholanthrene (3MC) (Chao Yeh et al., 1992; Fardel et al., 1996),and 2) ABCC1, ABCC2 and ABCG2 are known to efflux mainly phase II metabolites, xenobiotics conjugated with GSH, GA and/or sulphate (for a review see Leslie et al., 2005). These results have also strengthened the cooperative role of ABC efflux transporters and biotransformation enzymes in xenobiotic breakdown and elimination.

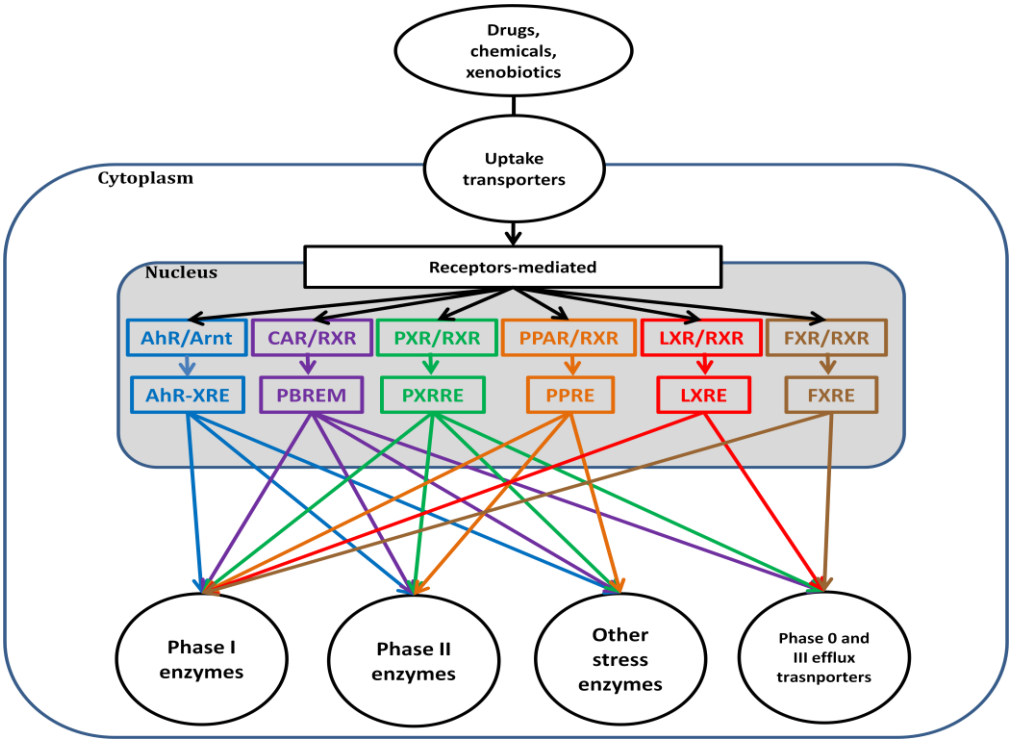

Figure 1.5 - Schematic representation of drugs/chemicals/xenobiotics induced stress response, leading to the activation of specific receptor-mediated gene expression of phase I enzymes, phase II enzymes, other stress enzymes and phase 0 and phase III transporters, which result in the enhancement of xenobiotic detoxification.

Ahr, aryl hydrocarbon receptor; Arnt, aryl receptor nuclear translocator; XRE, xenobiotic responsive element; CAR, constitutive androstane receptor; PBREM, phenobarbitol responsive enhanced module; RXR, retinoid X receptor; PXR, pregnane X receptor; PXRRE, pregane X receptor responsive elements; PPAR, peroxissome proliferator activated receptor; PPRE, PPAR responsive element; LXR, liver X receptor; LXRE, liver X receptor responsive element; FXR, farnesoid X receptor; FXRE, farnesoid X receptor responsive element. Adapted from Xu et al. (2005).

Thus, considering these characteristics of common regulation mechanisms and complementarity of substrates, it is believed that ABCB1 acts as a first line of defence preventing unmodified compounds from accumulating in the cell (phase 0 of cellular detoxification), while ABCCs and ABCG2 transport phase I and II metabolites, therefore acting in phase III of cellular detoxification (Bard, 2000; Sturm and Segner, 2005) (figure 1.6). Nevertheless, in aquatic organisms, only a few studies have been conducted in an attempt to elucidate this cooperative mechanism (Cooper et al., 1999; Sturm et al., 2001; Bard et al., 2002b; Paetzold et al., 2009). The majority of these studies have been confined to the study of Pgp and CYP1A, and results suggest that these two proteins should not be coordinately regulated in fish, but enhance the fact that they may act in complementarity in cellular detoxification. However, the database on the relation between Pgp and biotransformation enzymes in fish is small and dispersed, and the role of other ABC efflux transporters, besides Pgp, is even more poorly documented. Thus, the full characterization of this mechanism of cellular detoxification, where ABC efflux transporters and biotransformation enzymes display title-roles is yet far from being completely understood. Therefore, further studies are still needed to enlighten the underlying basis of these mechanisms of cellular detoxification, since this additional knowledge has implications for environmental, but also for human health.

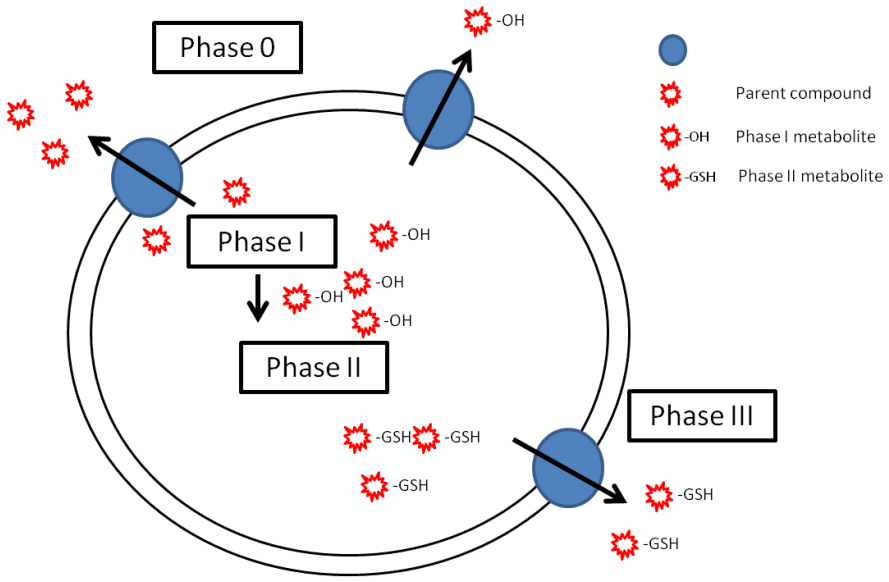

Figure 1.6 – Schematic representation of the possible cooperation of ABC efflux transporters (phase 0 and phase III) and biotransformation enzymes (phase I and phase II) in cellular detoxification (adapted from Bard et al., 2000)

The medical relevance of ABC transporters is evidenced by their MDR abilities and, under this point of view there is a fundamental need to know how to properly block the action of these transporters to assure the targeting efficacy of chemotherapeutic drugs. In addition, the study of ABC efflux transporters is also very important from an environmental point of view, since they are believed to an integral part of the cellular detoxification system. For environmentalists, the goal is to understand how these efflux transporters keep toxicants out of the cells, and to ensure that they operate optimally in order to protect the cells from environmental contaminants.

1.6. PAHs (Polyciclic aromatic hydrocarbons)

Polycyclic aromatic hydrocarbons (PAHs) are prevalent environmental contaminants commonly found in aquatic sediments and waters associated with urbanized estuarine and coastal areas (Meador et al., 1995). Although these contaminants can arise from natural sources, such as oil seeps, volcanoes and forest fires, anthropogenic sources as vehicle exhaust, power generation and oil pollution are the main causes for environmental PAH contamination (Latimer and Zheng, 2003). Structurally, PAHs are a large group or organic compounds with two or more fused aromatic rings, and those with fewer aromatic rings are more water-soluble than heavier PAHs, and therefore more bioavailable. PAHs are readily absorbed by fish and other aquatic animals upon exposure to contaminated food, water and sediments (Neff, 1985). Relative concentrations of PAHs in aquatic ecosystems are, generally higher in sediments, medium in aquatic biota and lower in water column. In the aquatic environment, the degradation of PAHs can occur by photo-oxidation and biological transformations (Valerio et al., 1984). In fish, biotransformation of PAHs occurs mostly in the liver, but also in the extra-hepatic tissues, like the gills and the intestine (Van veld et al., 1997). These compounds are easily metabolized by phase I enzymes of the MO into more hydrophilic products like phenols, dihydrodiols, quinines and epoxides (Lech and Vodicnik, 1985). Some of the PAHs can be excreted directly as unconjugated polar metabolites in bile, but most of them will only be excreted after conjugation by phase II enzymes (van der Oost et al., 2003). The major PAHs-metabolization pathway involves CYP MO, epoxide hydroxilase and several conjugating enzymes. Several hundreds of different PAHs exists, and one of the best known is Benzo(a)pyrene (BaP). BaP is a

benzopyrene formed by a benzene ring fused to a pyrene, and is a result of incomplete combustion processes. This compound was classified as a group 2A carcinogen (probably carcinogenic to humans) by the International Agency for Research on Cancer, Word Health Organization (IARC, WHO) and, due to new information regarding its effects, was recently upgraded to a group 1 carcinogen (carcinogenic to humans). Additionally, BaP is one of the 16 PAH selected by the US Environmental Protection Agency (EPA) as priority pollutants, due to its carcinogenic and mutagenic properties (Agency for Toxic Substances and Disease Registry - ATSDR 1995). In addition, BaP is also one of the most commonly found PAHs in aquatic environments, and its toxicity to aquatic biota has been intensively studied and demonstrated (James et al., 1991; Reynaud and Deschaux, 2006; Amanuma et al., 2008), becoming a model compound for the study of PAHs biotransformation (Buhler and Williams, 1989; Stegeman and Hahn, 1994). BaP is a procarcinogen, meaning that its carcinogenicity is dependent of the enzymatic metabolism that can originate toxic electrophilic metabolites, with the ability to covalently bind to macromolecules, such as DNA and proteins (James et al., 1991; Cachot et al., 2004). BaP metabolism by phase I and II can follow different pathways, as described in figure 1.7. Some intermediates, such as phenols, quinones, and dihydrodiols are non toxic, as they can be detoxified by conjugation with phase II enzymes (IARC, 1983). However, besides conjugation, the dihydrodiols can undergo further oxidative metabolism, originating more toxic metabolites, as for example 7,8-dihydrodiol-9,10-epoxide, a compound considered to be the ultimate carcinogenic metabolite of BaP (Jerina and Dansette, 1984; Schlenk et al., 2008).

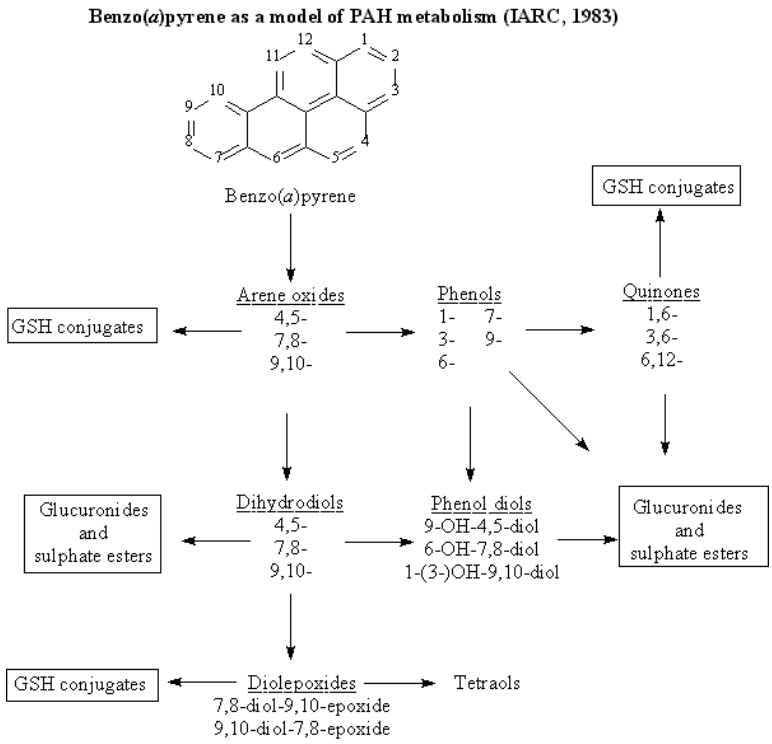

Figure 1.7 - Biotransformation pathways of BaP. Source: IARC, 1983

BaP was chosen as the model contaminant in this study, since its properties and effects on aquatic biota have been widely studied, and is representative of one of the most toxic group of pollutants commonly found in aquatic environments. Moreover, BaP is readily metabolized by phase I and II biotransformation enzymes, and it was proposed that the parent compound and/or its metabolites could have high affinity for ABC efflux transporters (Chao Yeh et al.,1992; Bard, 2000), thus gathering important characteristics for its selection as the model pollutant to assess the cooperation between efflux proteins and biotransformation enzymes in this study.

1.7. Model species – *Oreochromis niloticus* (Nile tilapia)

Fish offer many advantages for investigating the organism-environment interface due to their intimate physiological contact with the surrounding environment, and because they are sensitive sentinels of environmental challenge, particularly pollution. Additionally, their extraordinary diversity allows responses to the environment to be mapped onto an extensive phylogeny that extends over 500 million years of history,

providing insights into a range of evolutionary processes, from ancient to modern (Cossins and Crawford, 2005). In this study we have used the specie *Oreochromis niloticus*, Nile tilapia, as the model species (figure 1.8). Nile tilapia is an african endemic cichlid species, belonging to the order of perciformes. This tropical freshwater species lives in shallow waters, in temperature ranges from 31 to 36 °C, but it can tolerate lower (<12 ºC) or higher temperatures (>42ºC), and also higher salinities (up to 20ppt). It is an omnivorous grazer that feeds on phytoplankton, periphyton, aquatic plants, small invertebrates, benthic fauna, detritus and bacterial films associated with detritus. Presently, Nile tilapia is one of the most widely farmed freshwater fish in the world (Food and Agriculture Organization, FAO, 2006), having a high economical potential. Due to its easy handling, culture and maintenance in the laboratory, and because tilapia promptly responds to environmental alterations, this species is also a well-established model for research in a wide range of areas like toxicology (Almeida et al., 2001; Abdel-Tawwab et al., 2007; Coimbra et al., 2007), physiology (Wright and Land, 1998), endocrinology (Coimbra et al., 2005; Barreto and Volpato, 2007), genomic biology and molecular genetics (McConnell et al., 2000; Lee et al., 2005; Santini and Bernardi, 2005). Moreover, Nile tilapia is easy to maintain and reproduce in laboratory conditions since it has a high fecundity rate (hundreds of eggs in a clutch), year-round breeding capability (2–3-week spawning cycle), and a short generation period (half a year). Due to the high amount of studies conducted with Nile tilapia as a model, the biology and physiology of this specie is well characterized (Morrison et al., 2001; Morrison et al., 2003; Fujimura and Okada, 2007), and, recently, detailed genomic information of Nile tilapia has also became available (www.ensemble.org), opening new comparative approaches on gene function across vertebrate species.

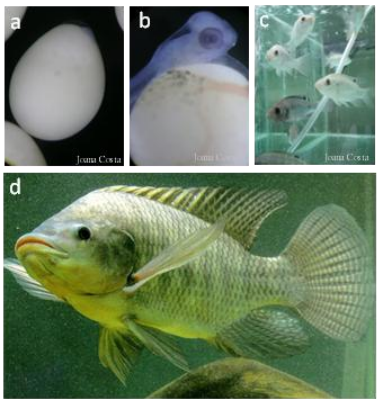

Figure 1.8 - Nile tilapia, Oreochromis niloticus, the model specie used in the present study.

a) embryos, b) larvae, c) juvenile, d) adult.

1.8. Objectives

Studies directed to the expression and/or co-expression of ABC efflux transporters and biotransformation enzymes are important to better understand the mechanisms of cellular detoxification, and this knowledge has implications to environmental and also to human health. Moreover, the role of ABC efflux transporters in the efflux of metabolized or unmodified compounds, and the cooperation with biotransformation enzymes is poorly documented in the different animal models, especially in aquatic organisms. In order to fill these gaps, the main goals of this PhD dissertation were the following:

1. to characterize the role of ecotoxicologically relevant ABC efflux transporters (ABCB1, ABCB11, ABCC1, ABCC2 and ABCG2) in the detoxification of PAHs in different barrier tissues of Nile tilapia;
2. to assess the cooperation between ABC efflux transporters and biotransformation enzymes of phase I and phase II in the detoxification pathway of PAHs in fish.

In order to achieve these goals, the following specific tasks were defined:

1) The *in vivo* assessment of BaP biotransformation mechanisms in Nile tilapia, after different routes of exposure to the contaminant (waterborne and dietary) through the evaluation of phase I and phase II catalytic activities and BaP metabolites formed.

2) The characterization of the expression, and response patterns, of ABC efflux transporters (ABCB1, ABCB11, ABCC1, ABCC2 and ABCG2) and biotransformation enzymes (CYP1A, GSTs and UGTs) in different barrier tissues of Nile tilapia (liver, gills and intestine) upon dietary and waterborne exposures to BaP. Characterization will be performed at gene level and, when possible, at protein (ABCB1 and CYP1A) and activity levels (CYP1A, GSTs, UGTs).

3) The characterization of the transcriptional patterns of ABC efflux transporters and biotransformation enzymes of phase I and phase II during the developmental stages of Nile tilapia.

The goals of this thesis were explored throughout the following chapters:

- Chapter 1 – General Introduction
- Chapter 2 - Comparision of the waterborne and dietary routes of exposure on the effects of Benzo(a)pyrene on biotransformation pathways in Nile tilapia (*Oreochromis niloticus*)
- Chapter 3 - Evaluation of 3-hydroxy-benzo[a]pyrene levels in Nile tilapia (*Oreochromis niloticus*) after waterborne exposure to Benzo(a)pyrene
- Chapter 4 - Gene expression analysis of ABC efflux transporters, CYP1A and GSTα in Nile tilapia after exposure to Benzo(a)pyrene
- Chapter 5 - Tissue distribution and response patterns of Pgp and CYP1A proteins in Nile tilapia (*Oreochromis niloticus*) after waterborne exposure to Benzo(a)pyrene (BaP)
- Chapter 6 - ABC transporters, CYP1A and GSTα gene transcription patterns in developing stages of the Nile tilapia (*Oreochromis niloticus*)
- Chapter 7 – Final remarks

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Chapter 2

Comparision of the waterborne and dietary routes of exposure on the effects of Benzo(a)pyrene on biotransformation pathways in Nile tilapia (*Oreochromis niloticus*)

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2. Comparision of the waterborne and dietary routes of exposure on the effects of Benzo(a)pyrene on biotransformation pathways in Nile tilapia (*Oreochromis niloticus*)

2.1. Abstract

BaP is one of the most studied PAH, due to its ubiquitous presence in aquatic environments and toxicity to aquatic organisms. The main goal of this study was to assess BaP effects in Nile Tilapia after waterborne and dietary exposures, through the evaluation of EROD and GST activities in liver, gills and intestine, and BaP metabolites in bile; and also to evaluate the usefulness of these commonly used biomarkers after two different routes of exposure. Waterborne exposure to BaP led to a significant induction of EROD in all tissues analyzed (644, 1640 and 2880% in relation to solvent in liver, gill and intestine respectively) while in dietary exposures EROD was induced only in intestine (3143%) after exposure to high BaP concentrations. GST activities with CDNB were slightly induced in liver (40%) and in gill (66%) after water exposure to BaP, and in intestine after dietary exposure to low BaP concentrations (182%). BaP metabolites in bile increased after both exposure routes, and were highly correlated with EROD activity after water exposure. In summary, this work has shown that the effects of BaP on biotransformation pathways depend on the route of exposure. Moreover, barrier tissues like gills and intestine also have an important role in the first-pass metabolism of BaP, reducing the amount of parent compound that reaches the liver to be metabolized. For that reason, EROD activity as a biomarker of exposure should also be applied in extrahepatic organs, like gills and intestine, in monitoring studies. Biliary BaP type metabolites are good reflectors of contamination levels under both exposure routes, while GST activity with CDNB as substrate, as a phase II enzyme, does not seem a reliable biomarker of exposure to BaP regardless the route of exposure.

Keywords: *Benzo(a)pyrene, biomarkers, tilapia, EROD, GST, bile metabolites*

2.2. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are prevalent environmental contaminants commonly found in aquatic sediments and waters associated with urbanized estuarine and coastal areas (Meador et al. 1995). Although these contaminants can arise from natural sources, such as oil seeps, volcanoes and forest fires, anthropogenic sources as vehicle exhaust, power generation and oil pollution are the main causes for environmental PAH contamination (Latimer and Zheng 2003). In aquatic organisms, the uptake of pollutants can occur through the contact of contaminated food, water and sediments (Bruggeman et al. 1984; Hendricks et al. 1985), and the degree of toxicity can be influenced by the route, by the dose and by the duration of exposure (Grimmer et al. 1988; Driver et al. 1991; Bloomquist 1992). One of the most common and toxic PAH in the aquatic environments is Benzo(a)pyrene (BaP) and its carcinogenic and mutagenic properties are widely studied (Buhler and Williams 1989; Tsukatani et al. 2003). The great majority of the studies focusing on BaP effects in the detoxification mechanisms in aquatic organisms have used intraperitoneal injections (Van Der Weiden et al. 1994; Pacheco and Santos 1998; Nacci et al. 2002; Wang et al. 2006; Wang et al. 2008; Nahrgang et al. 2009) as the route of exposure. Some studies have investigated the effects on the detoxification mechanisms of BaP after dietary (Hendricks et al. 1985; Wolkers et al. 1996; Reynolds et al. 2003), and waterborne exposure routes (Levine and Oris 1999; Wu et al. 2007; Ortiz-Delgado et al. 2008; Wang et al. 2008) which are two of the most important routes of pollutants uptake in aquatic organisms. However, to our knowledge, there is still a lack of studies that directly compare the biochemical effects of different concentrations of the same contaminant after both exposures routes in the same species.

In fish, as in other vertebrates, exposure to PAHs results in the induction of enzymatic systems involved in the metabolism of the xenobiotic compound, for the detoxification of the organism (Black and Coon 1987; Buhler and Williams 1989) due to a high-affinity of these pollutants to bind the aryl-hydrocarbon receptor (AhR). Studies in mammals have shown that the activation of the AhR-dependent signal transduction pathway controls the expression of several genes, including cytochrome P4501A (CYP1A) and some glutathione-S-transferase (GST) genes (Hankinson 1995, Schmidt and Bradfield 1996, George 1994). Like in mammals, in fish these genes are believed to be primarily involved in hepatic biotransformation of pollutants, like PAHs (reviewed in Van der Oost et al. 2003). The measurement of phase I and phase II biotransformation enzymes hepatic activities, have been widely applied as biomarkers of biochemical effects, and as a complement to chemical analysis (Ferreira et al. 2006, Ferreira et al. 2008, Richardson et

al. 2001, Nahrgang et al. 2009; Reis-Henriques et al. 2009, Jönsson et al. 2009). However, despite the fact that the liver is considered to be main site of CYP1A expression, its expression and induction has been observed in other tissues as well, including those in direct contact to the environment, such as gills (Jönsson et al., 2004, 2006, Nahrang et al., 2010a, 2010b) and intestine (James et al., 1997, McElroy and Kleinow, 1992). Nevertheless, the relative importance of branchial and intestinal uptake route to the overall exposure remains poorly understood. After PAH metabolism in the liver, their metabolites are secreted into the bile, stored in the gall bladder and excreted to the intestinal tract (Au et al. 1999). Therefore, due to their high metabolism rate in fish (Meador et al. 1995), PAHs tend not to bioaccumulate in tissues, and the direct quantification of these compounds in fish tissues may not yield an accurate estimation of contaminant exposure and uptake. As a result, the measurement of PAHs metabolites in fish bile is considered a reliable method for assessing the degree of exposure of the organisms to these types of pollutants (Krahn et al. 1986). In this study, Nile tilapia (Oreochromis niloticus) was used as model species. Besides being an economically important cultured species, namely in Asia and Africa, Nile tilapia is also a well established model in many toxicological studies (Almeida et al. 2001; Straus 2003; Coimbra et al. 2005, 2007; Figueiredo-Fernandes et al. 2006).

The main objectives of the present work were to evaluate differences in the detoxification mechanisms in juvenile Nile tilapia, after waterborne and dietary exposure routes to BaP. Biochemical effects of BaP were assessed, by means of 7-ethoxyresorufin O-deethylase (EROD) and glutathione-S-transferase (GST) activities as a measure of phases I and II of the biotransformation mechanism, in liver, gills and intestine. Additionally, fixed wavelength fluorescence (FF) was used to quantify BaP type metabolites as fluorescent aromatic compounds (FACs) in bile.

2.3. Materials and methods

2.3.1. Chemicals

Benzo(a)pyrene (99% purity), resorufin sodium salt, 7-ethoxyresorufin, ß-NADPH (95% purity), BSA (99% purity), 1-chloro-2,4-dinitrobenzene (CDNB, 97% purity), α-Dithiothreitol (99% purity), GSH (99% purity) were purchased from Sigma Aldrich, Germany. All the other chemicals were of analytical grade, and were purchased from local companies.

2.3.2. Animals

All animals used in this study were born and raised in the laboratory (CIIMAR, Porto, Portugal). The corresponding breeders stock was obtained from the Aquaculture Station of UTAD (Universidade de Trás-os-Montes, Portugal). Fish used in all assays were juveniles, and therefore not sexually mature, as confirmed by macroscopic analysis of the gonads when animals were sacrificed. Until the start of the exposure assays, fish were kept in 60 L aquaria supplied with biological filtration. Prior to the experiments, animals were randomly distributed in the experimental aquaria (30 L), and submitted to an acclimatation period of one week. All tanks were supplied with continuous aeration to maintain dissolved oxygen near saturation. Dechlorinated tap water was used at a temperature of 20 ± 2 ºC, with a 12h:12h (light:dark) photoperiod, and fish were fed commercial food pellets (Aquasoja, Portugal) until satiation, once a day.

2.3.3. Stock solutions of BaP and preparation of contaminated food

For the waterborne exposures, stock solutions of BaP were prepared in acetone (0.5, 1.25, 2.5 and 5 g/L) and were administered directly in the experimental aquaria. The percentage of solvent added in the experimental aquariums was 0.002%. For the dietary exposures stock solutions of BaP with concentrations of 0.1 and 40 g/L were prepared, respectively for the first and second assays. The contaminated diets were prepared by immersion of food pellets in BaP stock solutions diluted in acetone, in a proportion of 0.32 ml/g of food. For control groups, nothing was added to the food pellets, and for solvent control groups only acetone was added to the food pellets. Acetone was evaporated under air current for 24 hours, until the pellets were completely dry, and diets were stored at -20 ºC until further use.

2.3.4. Xenobiotic exposures

For the water exposure, juvenile Nile tilapia (N=116, average weight of 14.2±0.6 g, average length of 9.30±0.14 cm) were exposed to nominal water concentrations of 10, 25, 50 or 100 µg of BaP/L for 14 days. Also, a control group and a solvent control group (only solvent was added) were maintained. All treatments were performed in duplicate, with two exposure tanks per concentration. Waterborne exposures were conducted in semi-static conditions in 30 L aquaria. Daily, 80% of the water was renewed, followed by the addition of fresh BaP solution or solvent to each one of the treatment groups. Animals were fed to satiation every two days, with the exception of the day before sampling. Sampling was performed prior to the first BaP addition, and 7 and 14 days after the contaminant addition.

For dietary exposures two experiments were conducted, and fish were maintained in 30 L aquaria in continuous water flow conditions, which assured 100% of water renewal per day. In the first assay, juvenile tilapia (N=72, average weight of 11.21±1.00 g, average length of 8.47±0.27 cm) were exposed to 1 and 10 µg of BaP/g of food for 14 days. In the second dietary assay, fish (N=129; average weight: 11.15±0.42 g, average length of 8.49±0.09 cm) were exposed to 100 and 200 µg of BaP/g of food for 21 days. Fish were sampled before the addition of BaP contaminated diets, and at days 7, 14 and 21 (only in the second experiment). In both assays a control (uncontaminated food) and a solvent control group (acetone alone) were maintained, and duplicates were made for each treatment, with two exposure tanks per concentration. Fish were fed daily at a rate of 3% of body weight. After feeding, animals were observed, to assure that the total of the food was consumed in 2 or 3 minutes. After every sampling point, animals were weighted in order to recalculate the amount of food necessary for each tank, to maintain the percentage of feeding at 3% body weight.

2.3.5. Sampling

Fish were anesthetized on ice cold water and sacrificed by decapitation, and body weight and length were recorded for subsequent calculation of condition factor (CF = body weight (g) X 100/ body length^3 (cm)). Liver, gills and intestine were excised from the animal, and bile was collected from the gall bladder with a 1ml syringe. Liver weight was recorded for the calculation of hepatic somatic index (HSI = liver weight (g)/body weight (g) X100). Liver, gills, intestine, and bile were immediately frozen in liquid nitrogen, and stored

at -80ºC until further use. CF and HSI prior to the beginning of the water assay were 1.66±0.04 and 1.33±0.11, respectively. Before the dietary exposures, CF and HSI were 1.62±0.07 and 1.21±0.07 for the first assay, and 1.68±0.03 and 1.95±0.05 for the second assay, respectively. No significant differences were seen in these parameters during the course of the experiments, indicating that the overall condition of the animals was maintained during the experimental periods (Ricker 1975, Grant and Brown 1999).

2.3.6. BaP determination in water and food samples

To determine the real concentration of BaP in the water of the experimental aquaria, BaP was extracted from water samples according to the method described by Cheikyula et al. (2008), and assessed by the method of Rey-Salgueiro et al. (2008). After the addition of the contaminant, the real concentrations of BaP in water samples were 10.49, 22.51, 41.46 and 84.77 µg/L, respectively for the nominal concentrations of 10, 25, 50 and 100 µg/L. After 24 hours, and before the addition of fresh contaminant to the aquaria, the amount of BaP still present in each one of the treatment groups was 0.34, 0.69, 12.19 and 25.70 µg/L. In water collected from control and solvent control groups no BaP was detected at both times.

Food pellets supplied to the fish in dietary exposures were also analyzed in terms of BaP concentration, according to the method described by Rey-Salgueiro et al. (2009). The real BaP concentrations in the food pellets were 0.06, 0.07, 0.42, 3.99, 36.24 and 92.70 µg/g, respectively for control, solvent, 1, 10, 100 and 200 µg/g.

2.3.7. Biochemical analysis

EROD activity was measured according to Ferreira et al. (2008) in the microssomal fraction of liver, gills and the first one-third of the intestine. Briefly, tissues were homogenized in ice cold buffer (50 mM Tris-HCl, 0.15 M KCl, pH 7.4), and microsomes were obtained by centrifugation of the 9000 g supernatant at 36000 g for 90 minutes at 4 ºC. The pellet was then ressuspended in buffer (50 mM Tris-HCl, 1 mM Na2EDTA, 1 mM dithiothreitol, 20% v/v glycerol pH 7.4) and spun down at 36000 g for 120 minutes (Bucheli and Fent 1995). Microsomes were suspended in EDTA-free resuspension buffer and stored at -80 ºC until use. Microsomal suspension (20 µl), with average protein concentration of 3.01±0.20 mg/ml, was incubated with ethoxyresorufin (2 µM in 50 mM

Tris-HCl, 1 mM dithiothreitol, 0.15 M KCl, pH 8.0) for 1 minute, and the enzymatic reaction was initiated by the addition of NADPH 4.5 µM. EROD activity was measured for 5 minutes at λex530 nm and λem585 nm, by fluorometry, and determined by comparison to a resorufin standard curve (concentrations range from 3 to 100 nM). Hepatic EROD activity was expressed in pmol/min/mg protein. During the procedure of microsomes isolation, an aliquot of the cytosolic fraction (obtained after the 9000 g centrifugation) was separated for the analysis of GST activity. GST was determined according to the method of Habig et al. (1974), adapted to microplates as described in Ferreira et al. (2008), using glutathione (GSH) 10 mM in phosphate buffer 0.1 M, pH 6.5, CDNB 60 mM in ethanol prepared just before the assay. The reaction mixture was composed by 1.5 mM GSH and 1.5 mM CDNB in phosphate buffer. In the microplate, 0.2 ml of the reaction mixture was added to 0.1 ml of sample, corresponding to 0.3 mg of protein, with a final concentration of 1 mM GSH and 1 mM CDNB in the assay. The GST activity was measured immediately every 20 seconds, at 340 nm, during the first 5 minutes, and calculated in the period of linear change of the absorbance. GST activity was expressed in nmol/min/mg protein. In each treatment group, EROD and GST activities were analysed in 4-10 individuals, depending on the tissue. Protein content in all assays was determined by Lowry method (Lowry et al. 1951), in the correspondent fraction.

2.3.8. BaP metabolites in fish bile

BaP metabolites in the bile were determined through fixed wavelength fluorescence (FF). Bile samples from control and solvent control of water exposed animals, and from food exposed groups were diluted 1:1000 in ethanol 48%. Bile from water exposed animals was further diluted to 1:10000 (10 and 25 µg/L) and 1:100000 (50 and 100 µg/L). FF was performed at the excitation/emission wavelength pair 380/430 nm, since BaP metabolites are more efficiently detected at these wavelengths (Krahn et al. 1993, Lin et al. 1996). Measurements were performed on a BIOTEK SFM25 fluorimeter. The FF values were expressed as arbitrary fluorescence units (a.f.u.) after subtracting the signal levels of the solvent. The bile pigment biliverdin was measured at 380 nm in all samples to estimate bile density, and BaP metabolites fluorescence was normalised to biliverdin, to check if differences in fluorescence intensity could result from differences in bile densities. BaP metabolites were analysed in bile of 4-10 individuals of each treatment group.

2.3.9. Statistical analysis

Exposure time and treatment effects were evaluated by means of a one-way ANOVA for each one of the mentioned factors, followed by a multiple comparision test (Tukey´s test) at a 5% significance level. Some data had to be log transformed in order to fit ANOVA assumptions. Correlations and all the tests were performed using the software Statistica 7 (Statsoft, Inc., 2004). No differences were observed between replicates of the same treatment, nor in control treatments between days, nor between control and solvent control during the exposure time. Therefore, in all biomarkers analysed, results were presented as percentage in relation to solvent control only. Results were shown as mean±standard error.

2.3.10. Ethics statement

The animals used in the research that is described in this paper were treated in accordance with the Portuguese Animals and Welfare Law (Decreto-Lei nº 197/96) approved by the Portuguese Parliament in 1996.

2.4. Results

Mean control values of EROD activity in all assays were 38.09±3.85, 2.09±0.23 and 1.10±0.10 pmol/min/mg of protein, respectively for liver, gill and intestine. GST had mean activities in control groups of 120.79±4.46, 63.96±3.51 and 49.16±3.73 nmol/min/mg of protein, in liver, gill and intestine, respectively. BaP metabolites in bile were 4584±410 a.f.u. in control animals.

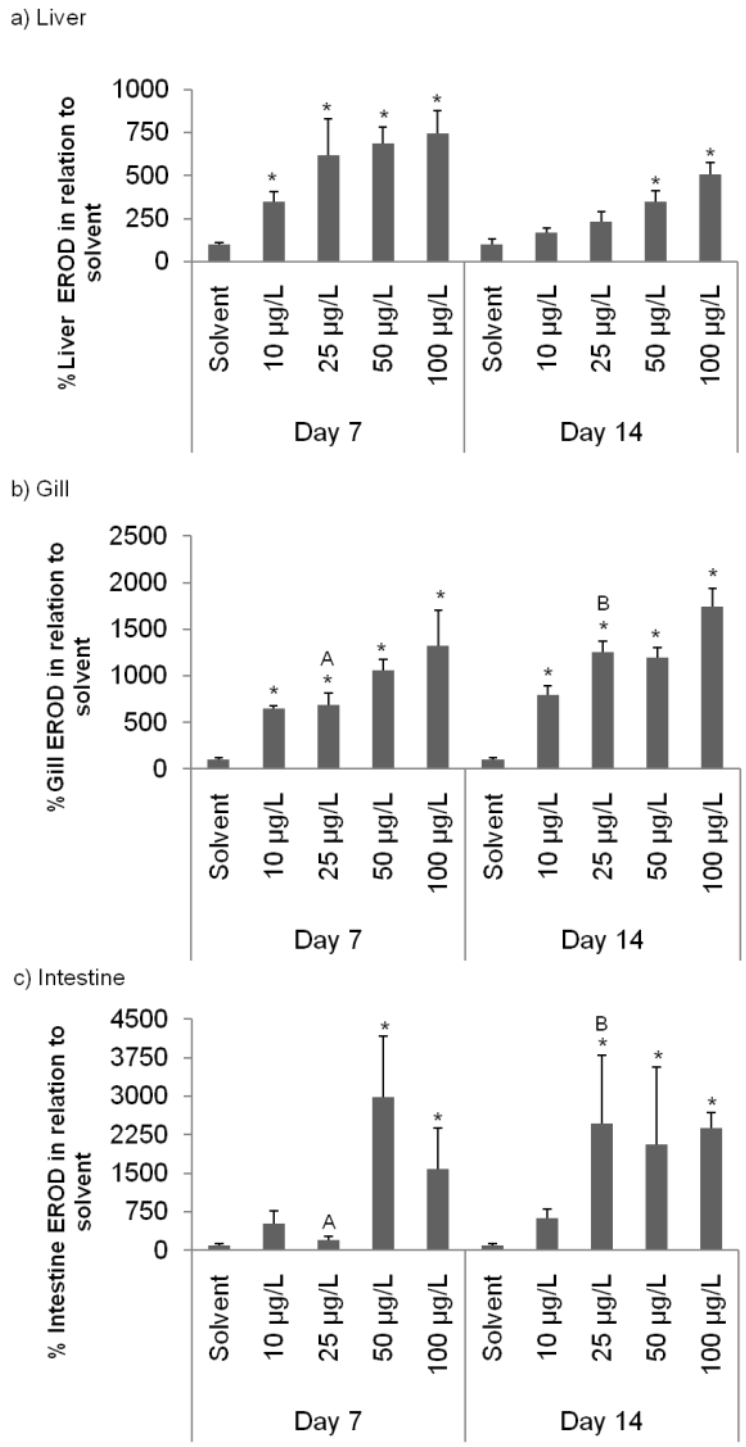

Figure 2.1 - EROD activities in liver (a), gill (b) and intestine (c) of Nile Tilapia after waterborne exposure to BaP (Solvent, 10, 25, 50 and 100 µg/L).

Values are expressed in percent of activity (pmol/min/mg of protein) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (*) denote significant differences ($p<0.05$) in comparison to solvent. Capital letters denote significant differences ($p<0.05$) between days within the same treatment. n = 4-10.

2.4.1. Water exposure

Biomarkers evaluated after waterborne exposure to BaP are shown in figures 2.1 (EROD activity), 2.2 (GST activity) and 2.3a (BaP metabolites in bile).

Results show that water exposure caused BaP dose-related increases of EROD activity in liver (figure 2.1a), gills (figure 2.1b) and intestine (figure 2.1c). Moreover, EROD activity was, in most of the treatments, significantly different from solvent control ($p<0.05$) in all three tissues. Exceptions were exposure to 10 and 25 µg/L at day 14 in liver, and in intestine exposure to 10 µg/L (at days 7 and 14) and 25 µg/L (at day 7). Regarding the effects of time of exposure, a significant increase between days 7 and 14 was seen in gills and intestine after exposure to 25 µg/L of BaP. When comparing the extent of EROD activity increase over the solvent control, maximum increases were seen in intestine (from 105 to 2876% increase, figure 2.1c), followed by gills (from 544 to 1639% increase, figure 2.1b) and liver (from 70 to 644% increase, figure 2.1a).

Regarding phase II enzyme, GST, its activity, increased significantly ($p<0.05$) in liver (after 14 days of exposure – figure 2.2a) and gills (after 7 days of exposure – figure 2.2b) upon exposure to 100 µg/L of BaP (39 and 66% increases respectively). At day 7 no differences were seen in GST intestine activity, but after 14 of exposure its activity decreased significantly ($p<0.05$) after exposure to 50 and 100 µg/L, achieving a maximum decrease over solvent control of 64% after exposure to 100 µg/L of BaP (figure 2.2c).

BaP metabolites in bile of exposed animals (10, 25, 50 and 100 µg/L) were always significantly higher than the levels recorded in solvent, and increased with the concentration of the contaminant and with the time of exposure, with the exception of 100 µg/L at day 14 (figure 2.3a). Results showed that after 7 days of exposure to 10 µg/L, BaP type metabolites were 83 times higher when comparing to the solvent (8256% increase), and the maximum levels were seen after 14 days of exposure to 50 µg/L, with a 356 fold increase (35590%). Moreover, BaP metabolites showed significant positive correlations ($p<0.05$) with EROD activity in liver ($r=0.92$), gills ($r=0.89$) and intestine ($r=0.65$).

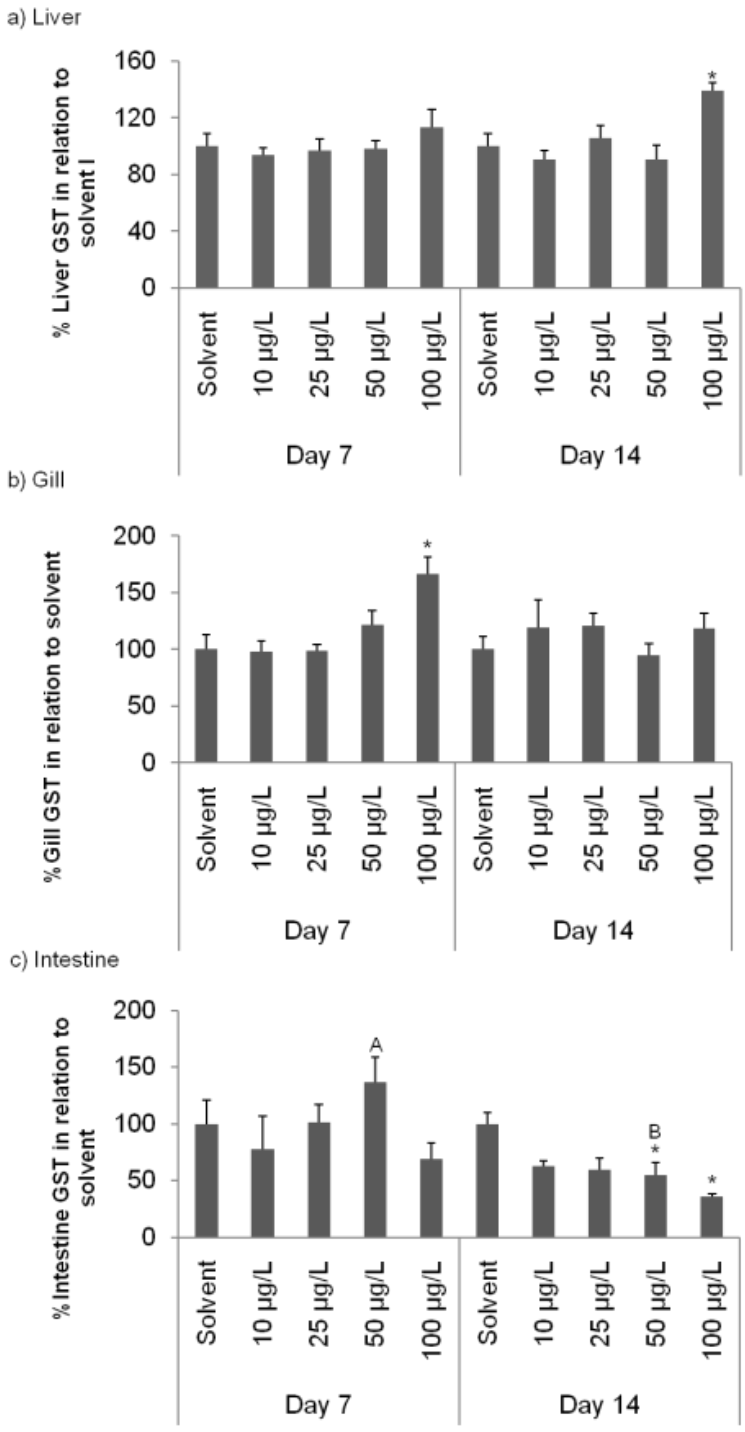

Figure 2.2 - GST activities in liver (a), gill (b) and intestine (c) of Nile Tilapia after waterborne exposure to BaP (Solvent, 10, 25, 50 and 100 µg/L).

Values are expressed in percent of activity (nmol/min/mg of protein) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (*) denote significant differences ($p<0.05$) in comparison to solvent. Capital letters denote significant differences ($p<0.05$) between days within the same treatment. n = 4-10.

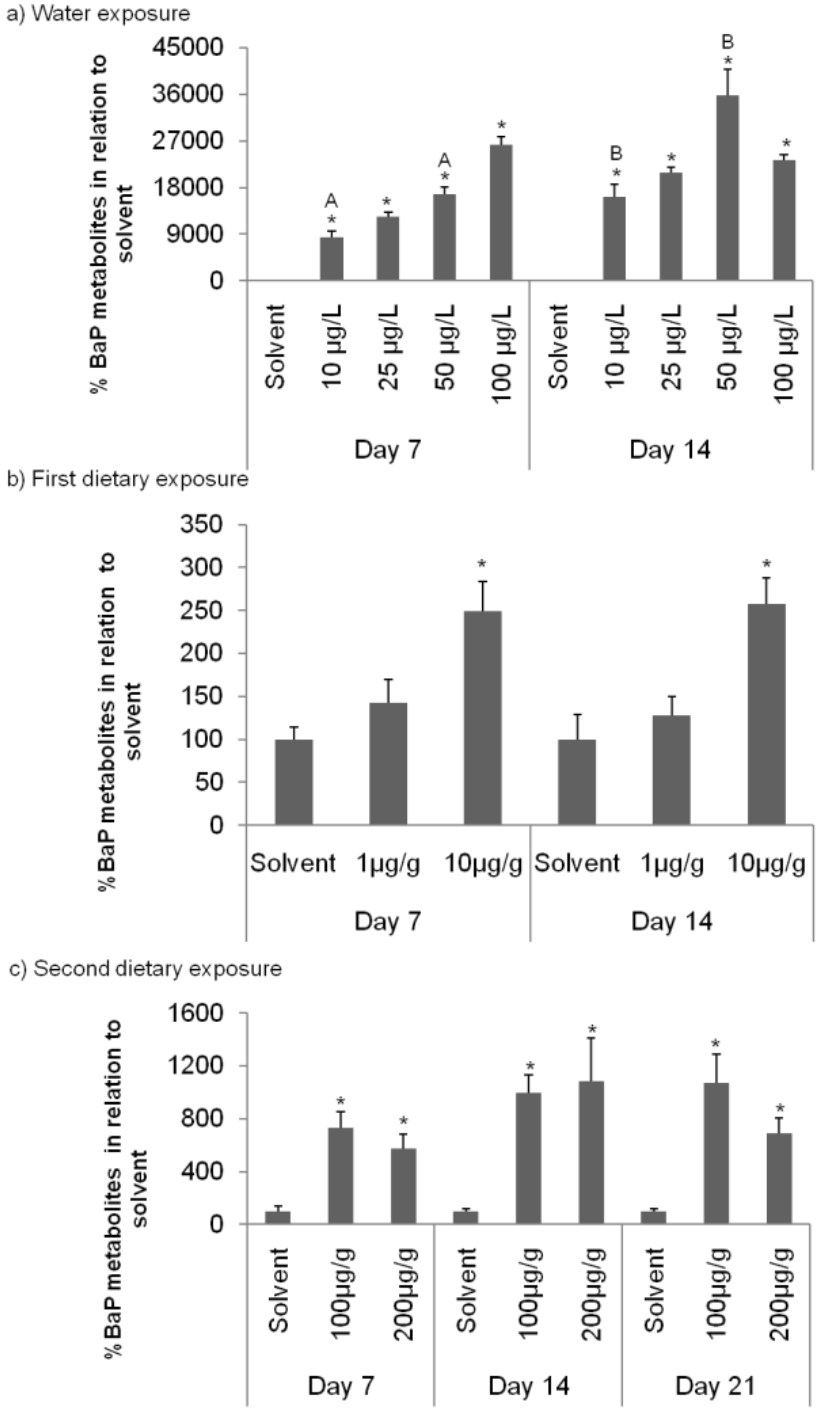

Figure 2.3 – BaP type metabolites in bile after waterborne exposure to BaP (a), first dietary exposure to BaP (b) and second dietary exposure to BaP (c).

Values are expressed percent of arbitrary units of fluorescence (a.f.u.) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (*) denote significant differences ($p<0.05$) in comparison to solvent. Capital letters denote significant differences ($p<0.05$) between days within the same treatment. n = 4-10.

2.4.2. Diet exposure

Results of EROD and GST activities in Nile tilapia after dietary exposure to 1 and 10 µg of BaP/ g of food are shown in figures 2.4 and 2.5, respectively.

EROD activity in exposed animals was not different from solvent control during the exposure periods in any of the tissues analyzed (figure 2.4). A significant decrease in EROD activity was seen from day 7 to day 14 at 1 µg/g of BaP in liver (figure 2.4a) and in gills (figure 2.4b). Phase II enzyme activity, GST, did not change in liver (figure 2.5a) nor in gill (figure 2.5b), and was only induced in the intestine, and after 7 days of exposure to 10 µg/g; however this induction was not sustained until day 14 (figure 2.5c). Regarding BaP metabolites in bile (figure 2.3b), BaP exposed groups showed higher levels when comparing to the solvent group, with significant differences at 10 µg/g of BaP at days 7 (149% increase) and 14 (158% increase) ($p<0.05$).

EROD and GST activities in liver, gills and intestine measured in the second diet experiment are displayed in figures 2.6 and 2.7, respectively.

In liver (figure 2.6a), results point to a pattern of EROD activity reduction after dietary exposure to 100 and 200 µg of BaP/g of food. This reduction was statistically supported at day 7 after exposure to 200 µg of BaP/g (45% decrease), and at days 14 and 21 to both BaP concentrations ($p<0.05$), with a maximum decrease of 68% after 14 days of exposure to 200 µg/g. Moreover, lower levels of EROD activity were detected in 200 µg of BaP/g of food than 100 µg/g at days 7 and 14, and at day 21 similar levels of EROD were recorded in both concentrations. Gill EROD activity did not change during the exposure to dietary BaP (figure 2.6b). However, a significant increase of intestinal EROD activity was seen in exposed animals during the course of the experiment, with a maximum increase occurring after 21 days of exposure to 100 µg/g (3142% increase over solvent control). Furthermore, there was a significant increase in intestinal EROD activity from day 7 to day 21 at this BaP concentration (figure 2.6c).

High dietary exposures caused no significant changes in GST activity in any of the tissues analyzed, as it can be seen in figure 2.7. BaP type metabolites (figure 2.3c) were always significantly higher in exposed animals (100 and 200 µg/g), with increases in relation to solvent ranging from 471% (200 µg/g, day 7) to 980% (200 µg/g, day 14).

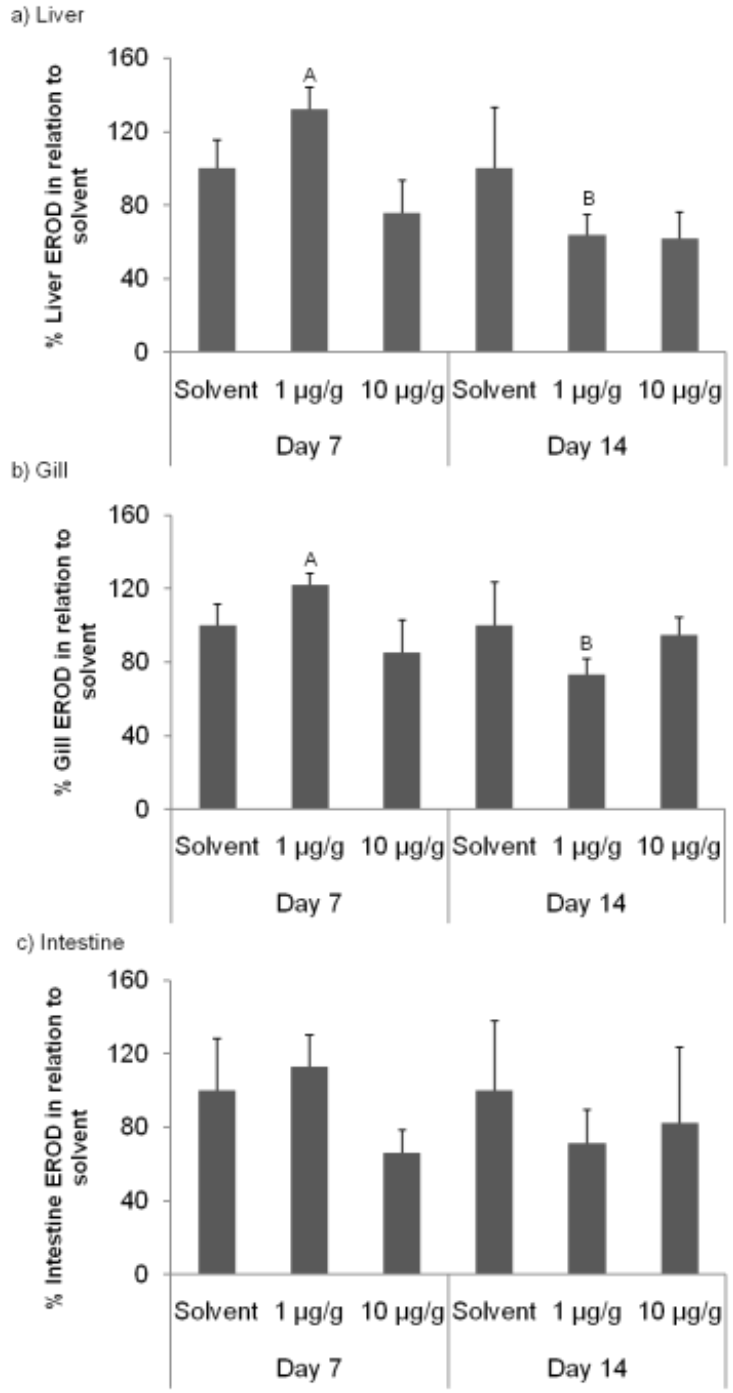

Figure 2.4 – EROD activities in liver (a), gill (b) and intestine (c) of Nile tilapia after the first dietary exposure to BaP (Solvent, 1 and 10 µg of BaP/g of food).

Values are expressed in percent of activity (pmol/min/mg of protein) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (*) denote significant differences ($p<0.05$) in comparison to solvent. Capital letters denote significant differences ($p<0.05$) between days within the same treatment. n=4-8.

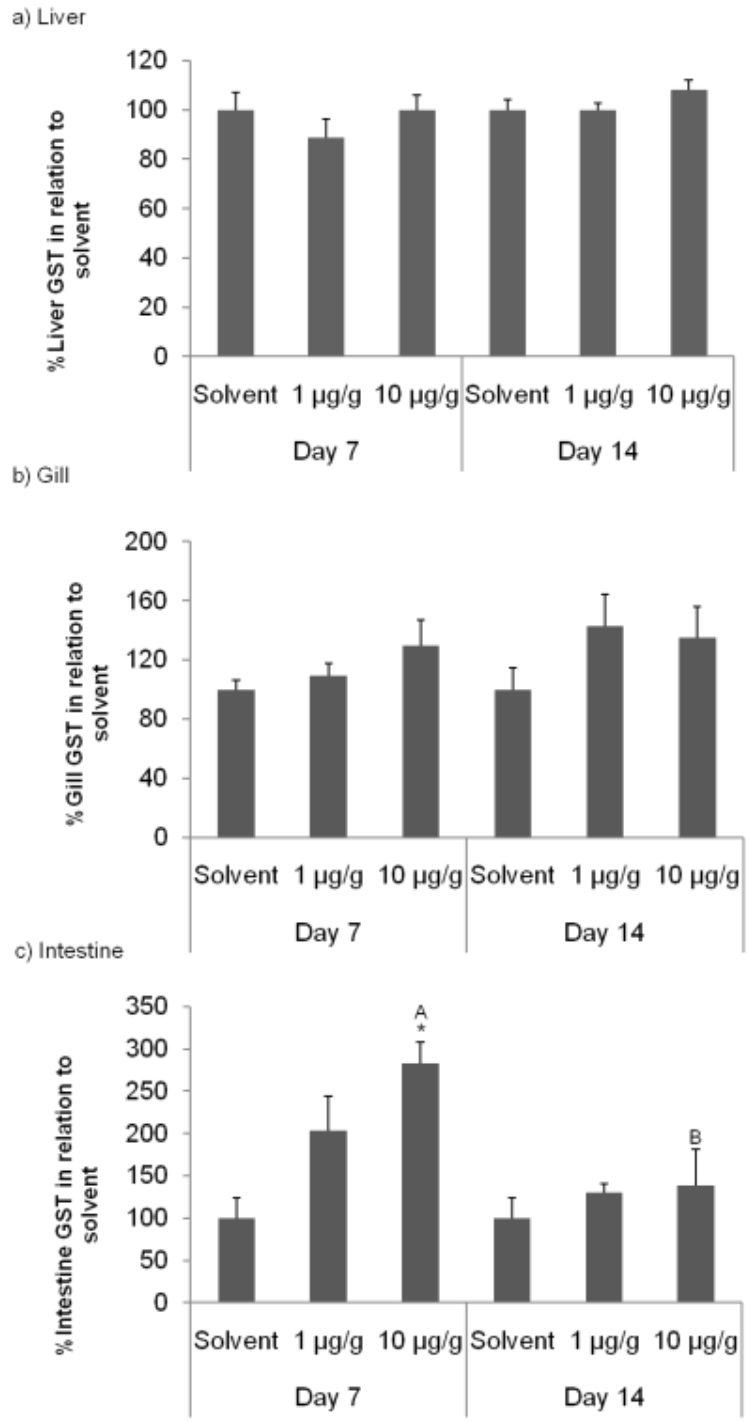

Figure 2.5 – GST activities in liver (a), gill (b) and intestine (c) of Nile tilapia after the first dietary exposure to BaP (Solvent, 1 and 10 µg of BaP/g of food).

Values are expressed in percent of activity (nmol/min/mg of protein) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (*) denote significant differences ($p<0.05$) in comparison to solvent. Capital letters denote significant differences ($p<0.05$) between days within the same treatment. n=4-8.

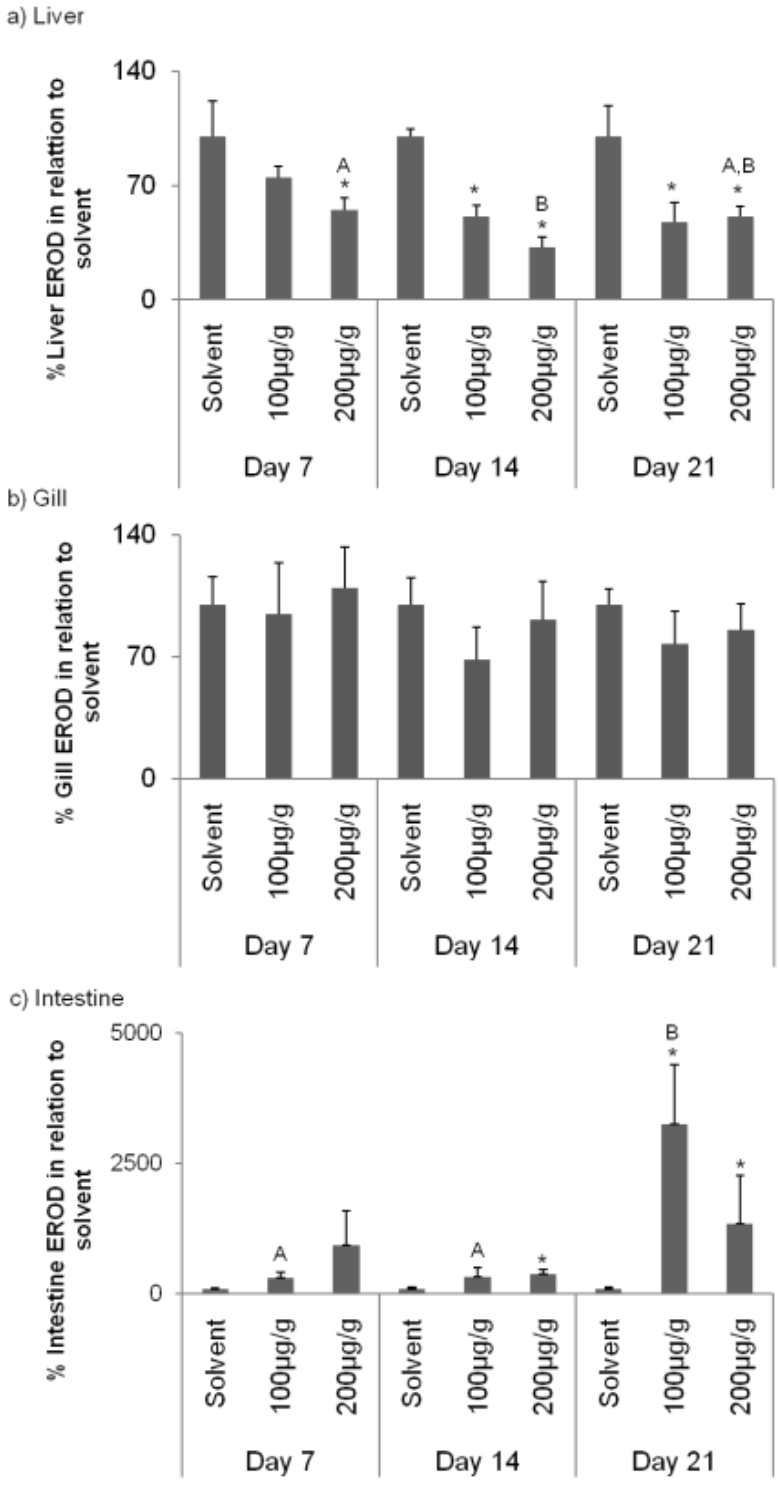

Figure 2.6 – EROD activities in liver (a), gill (b) and intestine (c) of Nile tilapia after the second dietary exposure to BaP (Solvent, 100 and 200 µg of BaP/g of food).

Values are expressed in percent of activity (pmol/min/mg of protein) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (*) denote significant differences ($p<0.05$) in comparison to solvent. Capital letters denote significant differences ($p<0.05$) between days within the same treatment. n=4-10

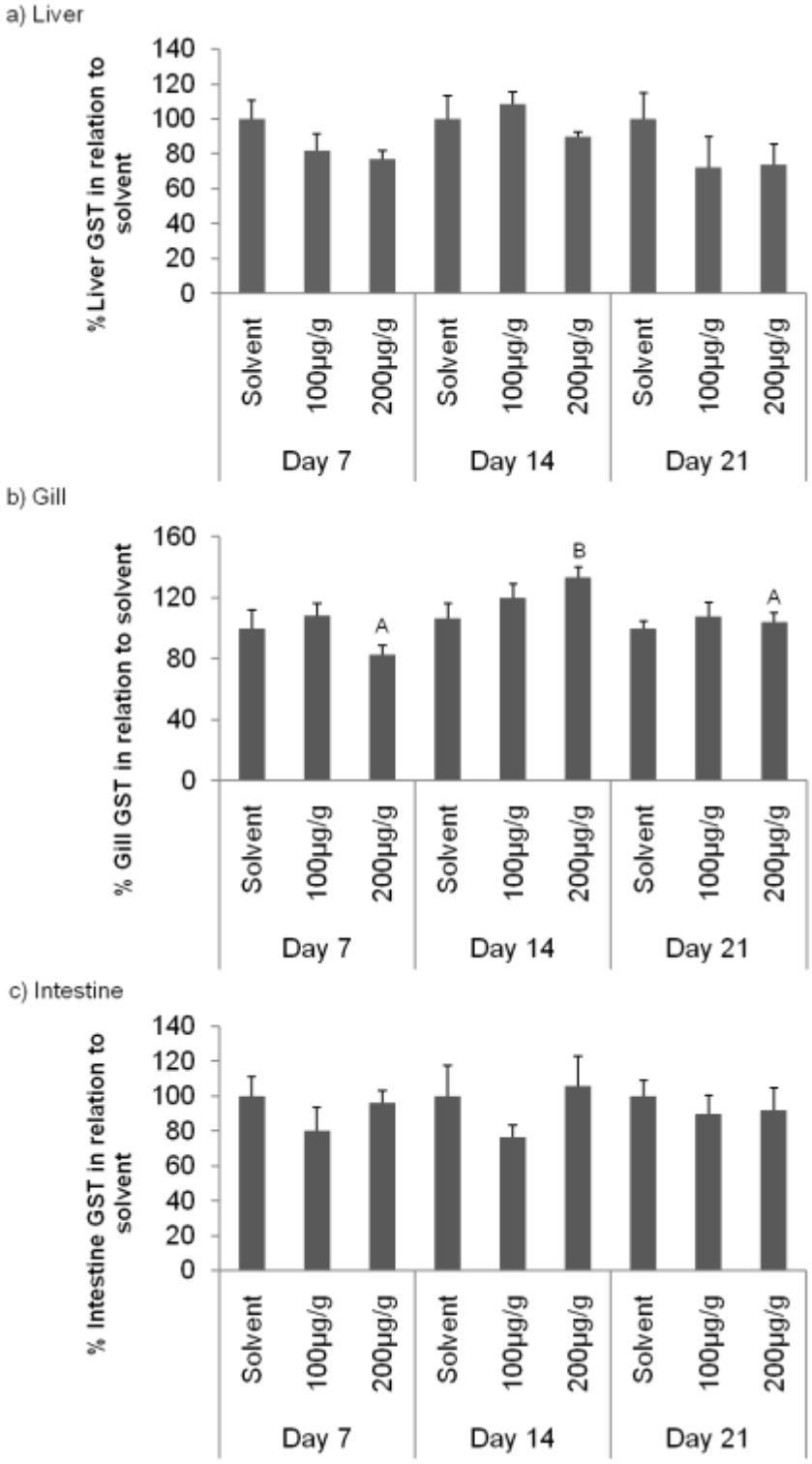

Figure 2.7 – GST activities in liver (a), gill (b) and intestine (c) of Nile tilapia after the second dietary exposure to BaP (Solvent, 100 and 200 µg of BaP/g of food).

Values are expressed in percent of activity (nmol/min/mg of protein) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (*) letters denote significant differences ($p<0.05$) in comparison to solvent. Capital letters denote significant differences ($p<0.05$) between days within the same treatment. n = 4-9.

2.5. Discussion

In recent years, there has been an increasing concern over the possible degradation of aquatic environments due to chemical pollution caused by PAHs, among other pollutants. The main reasons for that concern lie in the fact that these are widely spread contaminants with a high toxic potential for living organisms (Icarus Allen and Moore 2004). As a result, increased efforts have been made in order to understand the causal relationships between contaminant exposure and measurable biological and biochemical effects in aquatic organisms, with the integrated use of biomarkers of exposure. In the present study we aimed to achieve a deeper understanding of the effects of BaP in Nile tilapia, after two routes of exposure, waterborne and dietary exposures, and also to assess the usefulness of widely used biomarkers in monitoring studies.

After water exposure to BaP, EROD activity in liver (figure 2.1a), gills (figure 2.1b) and intestine (figure 2.1c) increased during the exposure period. These results show that waterborne exposure led to BaP metabolism in liver and also in extrahepatic tissues, as gills and intestine. Liver is considered to be the main metabolizing organ in fish, having larger amounts of CYP1A enzymes than other tissues (as gills and intestine) (Hinton et al. 2008), and induction of hepatic EROD activity upon exposure to PAHs has been described in other studies (Whyte et al. 2000; Van der Oost et al. 2003; Ortiz-Delgado et al. 2008; Kopecka-Pilarczyk and Correia 2009). This is in agreement with our study since hepatic EROD was strongly induced after exposure to waterborne BaP, and the levels of EROD activity were considerably higher in liver than in gill or intestine. The strong gill EROD induction observed after waterborne exposure to BaP also indicates that a rapid absorption of this compound occurs in this tissue, and supports the idea that gills work in the first-pass metabolism of BaP. Similar results, with gill EROD induction following BaP exposure, were obtained in other fish species as polar cod (Nahrgang et al.2010a) and rainbow trout (Jönsson et al. 2006). EROD induction observed in the intestine of BaP exposed animals was due to ingested contaminated water that was metabolized by intestine CYP1A enzymes, before reaching the liver. Although Nile tilapia is a freshwater specie, and therefore a specie who doesn´t drink water in large quantities, there is always some ingestion of water during feeding and breathing behaviors. Furthermore, our results are in agreement with immunohistochemical studies that compared the cellular expression of CYP1A in different tissues, and that showed that exposure to waterborne BaP resulted in high staining in gill pillar cells and hepatocytes and mildly staining in gut mucosal epithelium (Van Veld et al. 1997).

Some authors have shown that gill EROD activity turned out to be more sensitive than the liver in terms of CYP1A induction to waterborne BaP (Jönsson et al., 2006). The same happened in our study, with gill and intestine showing to be more sensitive than liver to the presence of BaP, which strengthens the idea that it becomes useful to measure EROD activity in extrahepatic tissues, that are involved in the first-pass metabolism of this compound (Levine and Oris, 1999).

After dietary exposures to BaP, the most significant result was the induction of EROD activity in the intestine, after exposure to the highest BaP concentrations (figure 2.6c). This type of response, with induction of intestinal but not hepatic EROD activity, have also been shown in other studies in liver (Reynolds et al. 2003, Van Veld et al. 1987) and intestine (Van Veld et al., 1987, James et al., 1997, McElroy et al., 1992) of fish exposed to dietary PAHs. Also, immunohistochemical studies showed that exposure to dietary BaP resulted in moderate CYP1A staining in liver but high intensity staining in gut mucosal epithelium (Van Veld et al. 1997; Ortiz-Delgado et al. 2005). We believe that, in our study, the absence of hepatic EROD induction following dietary exposures maybe directly related to partial BaP biotransformation in the intestine (by CYP1A enzymes), limiting the amount of parent compound that reaches the liver for hepatic metabolism, as confirmed by the induced intestinal EROD activity of these animals. However, in the second dietary assay, a reduction of hepatic EROD activity was seen after exposure to high BaP concentrations (figure2.6a), being statiscally significant at both concentrations at day 21. This situation could have been a result of protein degradation caused by the high concentrations of BaP used (reviewed in Whyte et al. 2001), or due to suppression of CYP1A at a post-transcriptional level mediated through a down regulation of the CYP1A protein (Schlezinger and Stegeman 2001). On the other hand, it is well known that BaP biotransformation can originate more toxic metabolites (Bauer et al., 1995; Kim et al., 1998, Morthy et al. 2003), and it is possible that part of this metabolites (formed mostly after intestinal CYP1A metabolism) could have entered systemic circulation (Kleinow et al., 1998), causing toxic effects at hepatic level that may have reflected in the lower EROD activities of this tissue. However, although the decrease of hepatic EROD activity was statistically significant, it was relatively weak, leading us to also consider the hypothesis of not being physiologically relevant.

BaP type metabolites measured in bile, increased after water exposure to BaP (figure 2.3a), and were highly correlated with liver ($r=0.92$; $p<0.05$), gill ($r=0.89$; $p<0.05$) and intestine ($r=0.65$; $p<0.05$) EROD activities. The liver correlation corroborates the fact that exposure of fish to waterborne BaP, leads to a pathway of high hepatic metabolism by CYP1A enzymes. Moreover, correlations between EROD and bile metabolites in

extrahepatic tissues also suggest that the majority of BaP metabolites formed in gill and intestine are reabsorbed into the blood stream and then released in the gall bladder. Regarding BaP type metabolites in the dietary experiments, an increase was also seen in BaP exposed animals (figures 2.3b and 2.3c), that was correlated with intestinal EROD activity ($r=0.62$, $p>0.05$), but not with hepatic EROD activity. Taken together these results indicate that, probably, dietary BaP metabolites formed in the intestine were reabsorbed into the blood for enterohepatic circulation and then excreted in the bile, as shown by Kleinow et al. 1998. Although we cannot compare the real amount of BaP that is metabolized under each one of the routes of exposure, it seems also important to highlight the fact that, levels of BaP metabolites were much more elevated after water exposure (achieving a fold increase of 356 times over solvent) than after dietary exposures (achieving a fold increase of 10 times over the solvent), despite the very high levels of BaP used after both exposures routes. These results suggest that, in field studies, the levels of biliary metabolites may give an indication of the main route of exposure to the contaminant.

Results obtained for phase II enzyme GST were not very clear, since in the case of changes in activity after BaP exposure, only slight variations were seen. The data available in the literature regarding GST use as a biomarker of exposure to pollutants is not very consistent, since some authors reported increases in this enzyme activity, while others did not observe any changes or even reported considerable reductions in its activity after exposure to PAH contaminants (reviewed in Van der Oost et al. 2003). Induction of GST activity was reported after waterborne exposure to BaP of *P. microps* (Vieira et al. 2008) and *S. marmoratus* (Wu et al. 2007). When it comes to dietary exposures to contaminants, some authors reported inductions of intestinal GST activities, but no induction of hepatic GST (Van Veld et al. 1991; James et al. 1997), suggesting a complementary role with phase I EROD in biotransformation of contaminants. Our findings corroborate the idea that phase II enzyme GST with CDNB as substrate has low sensibility to the presence of BaP, and should not be applied per se, as a biomarker of exposure to this pollutant. However, on the basis of substrate specificity, immunological cross reactivity and protein sequence data, mammalian citosolic GSTs have been grouped into seven classes: Alpha, Mu, Pi, Theta, Sigma, Omega and Zeta (Hayes et al., 2005) and previous publications have considered that the GST isoforms have distinct affinities towards the substrate CDNB (Hoarau et al. 2002; Martinez-Lara et al., 1996). Moreover, some studies have shown that, in fish, different responses are seen in GST isoforms after exposure to xenobiotics (Kim et al., 2010; Pérez-Lopez et al., 2002; Martinez-Lara et al., 1996), with some showing inducing patterns, while others do not. Therefore it is possible that, if some isoforms were

upregulated and others downregulated due to BaP exposure, the net result could be no change on total GST activity, which can possibly mean that the lack of changes in GST activity after BaP exposure does not necessarily indicate that this compound had no effect on GST activity.

In conclusion, this study has shown that the disposition and effects of BaP in biotransformation pathways in Nile tilapia depend on the route of exposure to the contaminant. Waterborne exposure to BaP resulted in an induction of phase I enzyme EROD in liver, gill and intestine, while in dietary exposure route induction of EROD was only seen in intestine, and after exposure to the highest concentrations of the pollutant. Therefore, EROD activity is a reliable biomarker of exposure to BaP in Nile tilapia and, besides liver, barrier tissues, like gills and intestine, should also be considered in biomonitoring studies. BaP metabolites are good reflectors of exposure to BaP, despite the route of exposure, and the levels of metabolites can also be indicative of the route of exposure, since water exposure leads to much higher levels of the metabolites than dietary exposure. The activity of phase II enzyme GST with CDNB does not seem as a reliable biomarker of exposure to BaP regardless the route of exposure.

2.6. Acknowledgements

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Chapter 3

Evaluation of 3-hydroxy-benzo[a]pyrene levels in Nile tilapia (*Oreochromis niloticus*) after waterborne exposure to Benzo[a]pyrene

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3. Evaluation of 3-hydroxy-benzo[a]pyrene levels in Nile tilapia (*Oreochromis niloticus*) after waterborne exposure to Benzo[a]pyrene

3.1. Abstract

3-hydroxy-benzo[a]pyrene (3-OH-BaP), a toxic compound with the ability to covalently bind to macromolecules (proteins and DNA), is one of the major phenolic metabolites of benzo(a)pyrene (BaP). The purpose of this study was to evaluate the presence of 3-OH-BaP in bile and plasma of Nile tilapia by HPLC with fluorescence detection, after waterborne exposure to BaP (10 and 100 µg L-1). Metabolites were detected in bile and plasma, and conjugates of 3-OH-BaP (glucuronide and/or sulphate conjugates) were the majority forms in both biological fluids, being the glucuronide 3-OH-BaP the main metabolite in bile. Our data suggest that extrahepatic tissues as intestine or gill are important in BaP metabolism and should be considered sources of metabolites released into the blood. Although, low levels of 3-OH-BaP in toxic form (free form) were detected in plasma, we must not exclude the possibility of circulating levels leading to toxic effects.

Keywords: *PAHs, metabolites, plasma, fish, bile, HPLC-fluorescence*

3.2. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants present in the marine environment as a result of natural sources, such as incomplete combustion of organic material, but mainly arising from human activities, as uncontrolled spills, river transport, surface runoff and atmospheric deposition (Latimer and Zheng 2003). Benzo(a)pyrene (BaP), one of the 16 PAHs selected by the US Environmental Protection Agency (EPA) as priority pollutants, is probably the most thoroughly studied and the most representative of this class of contaminants due to its carcinogenic and mutagenic properties (ATSDR 1995; IPCS 1998).

Fish can absorb BaP through the body surface, the gills and by ingestion of contaminated sediment or food, being distributed to tissues by the blood flow (Di Giulio and Hinton 2008; Fragoso et al. 2006; Varanasi et al. 1989). Upon exposure, fish have a well-developed system that will rapidly metabolize BaP in two phases (Varanasi et al. 1989), in a complex mixture of quinones, phenols, dihydrodiols, triols and tretols (Kennedy and Tierney 2008; Willett et al. 2000; Zhu et al. 2008). In phase I, BaP is metabolized into hydroxylated derivatives (OH-BaP) by the multienzymatic system cytochrome P450 (CYP450), being predominant the CYP 1A1 isozyme, although 1A2, 2A1 and 1B1 can also be active at a minor extent (Kim et al. 1998). In phase II, the OH-BaP metabolites are conjugated with polar endogenous constituents such as glucuronic acid, sulphate or glutathione by UDP-glucuronyl transferase, sulfotransferase and glutathione-S-transferase, respectively, to produce water-soluble conjugates that are easily excreted by fish (Ferreira et al. 2010; Tuvikene 1995). BaP metabolites produced in liver are secreted into the bile, concentrated and stored in the gallbladder before excretion into the intestine (Ferreira et al. 2006; Wang et al. 2008; Zhu et al. 2008). Although the liver is a major site of biotransformation of BaP, it can also occur in extrahepatic tissues as intestine, gills and kidney (Costa et al. 2011; James et al. 1996; Kleinow et al. 1998; Lemaire et al. 1990). Reactive metabolites produced in these tissues can be transferred to the blood for elimination but can also be re-distributed to other organs, interfering with their functions and, as a consequence, causing toxicity.

BaP itself is a relatively inert molecule, but their electrophilic metabolites are toxic, mutagenic, with the ability to covalently bind to macromolecules, such as proteins and DNA (Cachot et al. 2004; Fertuck et al. 2001; James 1991). One of the major phenolic metabolites of BaP formed in many species, including fish, is the 3-hydroxy-Benzo(a)pyrene (3-OH-BaP) which is produced in liver (Wang et al. 2008; Zhu et al. 2008)

and intestine (James et al. 2001). Due to toxic features of 3-OH-BaP, it should be more studied in the toxicological context, including it distribution to fish tissues. And, although, the blood plays an important role in the distribution of chemicals to organs, there is a lack of studies that measure BaP and metabolites in this tissue. Recently, BaP metabolism in extrahepatic tissues has been confirmed in Nile tilapia (Costa et al. 2011) which makes reasonable for the presence of metabolites in blood. Nile tilapia has been used in several toxicological studies and is considered a good model species to assess pollutants biological effects (Coimbra et al. 2007; Pereira Trídico et al. 2010). The main aim of the present work was to evaluate the presence of BaP and the metabolite, 3-OH-BaP, free and conjugated, in plasma and bile of Nile tilapia (*Oreochromis niloticus*) after waterborne exposure to BaP. Possible correlations with biotransformation enzymes in liver, intestine and gill, will be also assessed.

3.3. Material and methods

3.3.1. Chemicals and materials

Benzo(a)pyrene (99% purity), 3-hydroxy-Benzo(a)pyrene, tert-butyl hydroquinone (97% purity), 4-Methylumbelliferone, ß-glucuronidase from bovine liver and Uridine 5′-diphosphoglucuronic acid triammonium salt were purchased from Sigma Aldrich (Spain). ß-glucuronidase/aryl sulfatase from Helix pomatia was supplied by Merck (Germany) and Sep-Pak Plus C18 cartridges were obtained from Waters (USA). All the other chemicals were of analytical grade and were purchased from local companies.

3.3.2. Animals and exposure experiment

Nile tilapias used in this study were born and raised at CIIMAR, Porto, Portugal. Prior to the experiments, animals (N=112, average weight 70.3 ± 23.0 g) were randomly distributed (n=10) in the experimental aquaria (52 L) and submitted to an acclimation period of one week before the addition of the contaminant. All tanks were supplied with biological filtration, and continuous aeration to maintain dissolved oxygen near saturation. Dechlorinated tap water was used at a temperature of 21 ± 1 ºC with a 12 h:12 h (light:dark) photoperiod.

After the acclimatization period, fish were exposed to nominal water concentrations of 10 and 100 µg of BaP L^{-1} for 14 d. BaP stock solutions (1.04 and 5.20 g L^{-1}, respectively) were prepared in acetone and were administrated directly into the aquaria. The solvent concentration never exceeded 0.002%. A solvent control group (acetone alone) was also used. Experiment was conducted under semi-static conditions and daily 80% of the water was renewed, followed by the addition of fresh BaP solution or solvent to each one of the treatment groups. Animals were fasted for 48 h before each sampling, performed at 7 and 14 d.

3.3.3. Sampling

Water samples were collected, in triplicate, at the beginning (0 d) and at the end of the experiment (14 d) from two aquaria, at 0, 8 and 24 h after addition of the contaminant, for both BaP nominal concentrations.

Fish were anesthetized on ice cold water and blood was collected from the caudal vein using heparinized syringes. After blood collection, animals were sacrificed by decapitation. The blood was centrifuged at 4000 g for 7 min at 4 ºC and the plasma obtained was stored at -80 ºC until analysis. The liver, intestine and gills were excised from de animal, and bile was collected from the gall bladder with a 1 mL syringe, and were then immediately frozen in liquid nitrogen, and stored at -80 ºC until further use.

3.3.4. Biochemical analysis

Microsomal fractions of liver, intestine and gills were prepared as described in Fernandes et al. (2007). Briefly, tissues were homogenized in ice cold 100 mM KH_2PO_4/K_2HPO_4 (pH 7.4), containing 150 mM KCl, 1 mM dithiothreitol, 0.1 mM PMSF and 1 mM Na_2EDTA. Homogenates were centrifuged at 12000 g for 20 min and the resulting supernatant was further centrifuged at 100000 g for 60 min, to obtain the microsomal fractions. Microsomal pellets were resuspended in a ratio of 0.5 mL buffer g^{-1} of tissue in homogenization buffer containing 20% glycerol.

UDP-glucuronyl transferase (UGT) activity was assayed according to Collier et al. (2000). Fifteen µL of 20 mM uridine 5´-diphosphate glucuronic acid was added to a reaction mixture containing 15 µL of microsomal fraction and 120 µL of 125 µM 4-methylumbelliferone (4MU) in 0.1 M Tris-HCl containing 5 mM $MgCl_2$ and 0.05% BSA (pH

7.4) and incubated for 10 min at 37 ºC. Fluorescence was measured at λEX = 355 nm / λEM = 586 nm. Activities were expressed as pmol min^{-1} mg $prot^{-1}$ using a standard curve generated with 4MU (0.031-2.50 µM). Protein content in all assays was determined by Lowry method (Lowry et al. 1951), in the correspondent fraction.

3.3.5. Determination of BaP

The pre-analytical treatment for determination of BaP in water was performed according to the procedure for the extraction of PAHs in water previously described by Cheikyula et al. (2008), based on liquid-liquid extraction with n-hexane followed by HPLC analysis under the conditions described below.

For the analysis of BaP in bile and plasma, an analytical procedure similar to BaP extraction procedure in blood developed by Singh et al. (2008) was carried out. Bile (0.05 mL) and plasma samples (0.5 mL) were subjected to three consecutive ultrasound-assisted solvent extractions (UASEs) with 1 and 3 mL n-hexane, respectively, for 15 min each and the upper organic layer was collected and dehydrated with anhydrous sodium sulphate. The n-hexane extracts were evaporated to dryness under a gentle steam of nitrogen and the residue obtained was re-dissolved in methanol (0.1 and 0.2 mL for bile and plasma, respectively) and filtered through PTFE membranes (0.45 µm) for analysis (see section 3.3.8). Pooled samples were used (2-8 fish per pool, dependent on the volume of blood collected from each fish) for determination of BaP in plasma, while for bile, individual samples were analysed.

3.3.6. Determination of free and conjugates of 3-OH-BaP

The measurement of 3-OH-BaP in bile was performed according to the method of Ruddock et al. (2002), which included a prior enzymatic hydrolysis of the glucuronide and sulfate conjugated metabolites with a mixture of β-glucuronidase and aryl sulfatase (30 and 60 U mL^{-1} activity, respectively) or β-glucuronidase alone (1624 U mL^{-1}). Briefly, 10 µL of bile and 10 µL of enzyme solution were added to 230 µL of ultrapure water and the mixture was incubated at 37 ºC. After 45 min, the reaction was stopped by the addition of 250 µL of chilled methanol. After centrifugation (16000 g for 10 min) supernatants contain total 3-OH-BaP (T-3-OH-BaP) (conjugated and free 3-OH-BaP) after β-glucuronidase/aryl sulfatase hydrolysis or a mixture of glucuronide 3-OH-BaP conjugate (G-3-OH-BaP) and

free 3-OH-BaP after incubation with β-glucuronidase. For the determination of the free 3-OH-BaP, the analytical procedure was carried out without enzymatic hydrolysis and an extraction step was added. Bile (50 µL) was subjected to UASE with 100 µL methanol for 10 min and, finally, the extracts were also centrifuged and filtered for HPLC analysis (section 3.3.8).

In plasma samples, the pre-analytical treatment was based on procedures for the determination of T-3-OH-BaP in biological samples such as urine or milk (Rey-Salgueiro et al. 2009; Wang et al. 2005), with the extraction and enzymatic hydrolysis steps optimizated. To optimize the extraction step, plasma of non-exposed animals were spiked with 3-OH-BaP (10 µg L^{-1}) and extracted with different solvents (acetone, methanol, methanol:ethyl acetate (50:50)). Despite that the higher efficiency of extraction was obtained using methanol:ethyl acetate (97%), acetone (95%) was selected as the extraction solvent because of its higher vapor pressure, which facilitates the extraction step by being less time consuming. So, plasma samples (1 or 0.50 mL, for 10 and 100 µg L-1 exposure levels, respectively) were subjected to three consecutive UASEs with 2 x 1 x 1 mL acetone for 5 min each and the obtained extract was centrifuged (3000 g) for 3 min to facilitate separation of the liquid fraction, which was evaporated till dryness. To extract phase II metabolites from plasma samples, an enzymatic hydrolysis was conducted with a mixture of ß-glucuronidase/aryl sulfatase to obtain T-3-OH-BaP. Then, the obtained residues were filled up to a final volume of 50 mL buffer (0.10 M ammonium acetate buffer, 0.80 g L^{-1} tert-butyl hydroquinone and acid acetic until pH 5.5). The dissolved oxygen in the solution was then displaced with a nitrogen stream. The optimization of the enzymatic hydrolysis step was carried out directly in pooled plasma samples of exposed individuals to 100 µg L^{-1} BaP for 14 d. Volume of enzyme (10, 20 and 40 µL) and incubation time (0-16 h) were optimized for deconjugation and 20 µL was found to be adequate enzyme amount to de-conjugate 3-OH-BaP (data not shown). Deconjugation time, was optimized by adding 20 µL of enzyme solution and incubating at 37 °C. Conjugated metabolites were rapidly hydrolyzed in the first 2 h of incubation and slowly until 16h (data not shown), so an overnight deconjugation (16 h) was selected to be sufficient for hydrolysis of 3-OH-BaP in plasma samples. The hydrolysis conditions selected were in agreement with Fan et al. (2006) and Lutz et al. (2006), to enable hydrolysis of conjugated 3-OH-BaP in urine and milk samples, respectively. Afterwards, 5 mL methanol was added and the mixture was loaded onto a C18 sep-pack cartridge (previously activated with 5 mL methanol followed by 10 mL ultrapure water). Before the elution step with 10 mL methanol:ethyl acetate (50/50), the minicolumns were dried for 15 min under nitrogen stream at 15 bars. The eluate obtained was evaporated to dryness, re-dissolved in 0.20 mL methanol and filtered for HPLC analysis (section 3.3.8). For the determination of the free 3-OH-BaP, the

analytical procedure was carried out without enzymatic hydrolysis. Pooled samples were used (2-8 fish per pool) for determination of free 3-OH-BaP and T-3-OH-BaP in plasma. The number of animals per pool was dependent on the volume of blood collected from each fish. Due to the small amount of blood available, no G-3-OH-BaP was assessed in plasma.

3.3.7. Methods validation

To validate the analytical methods which were modified or optimized, plasma of non-exposed animals were spiked with BaP or 3-OH-BaP and were processed (n=5). The set of samples analyzed was processed with a blank to test for the background BaP and 3-OH-BaP levels in the material. Similar recovery rates of 3-OH-BaP, 59 and 60% with RSD lower than 6.80%, were obtained at two spiked concentration levels of 1 and 10 µg L-1, respectively, for the analytical method to measure 3-OH-BaP in plasma (table 3.1). Other methods based on solid phase extraction showed similar, 60-66% (Fan et al. 2006), and lower recoveries, <50% (Wang et al. 2005), in urine samples. Fan et al. (2006) suggested that this percentage of 3-OH-BaP is not due to the method, but to the instability of the compound in the acetate buffer, though it is stable in methanol. Therefore, the selected method was robust enough to quantify 3-OH-BaP in plasma samples. Detection and quantification limits (LOD and LOQ) were evaluated on the basis of the noise obtained with the analysis of unfortified blank samples (n=5) (Table 1). LOD and LOQ were defined as the concentration of the analyte that produced a signal-to-noise ratio of 3 and 10, respectively (ACS 1980) and were then tested experimentally by spiking blank samples at such levels. External standard calibration was used to quantify the samples by HPLC-FD technique using standard solutions of BaP and 3-OH-BaP (table 3.1).

Table 3.1 - Recoveries ± RSD, instrument linear dynamic ranges, determination coefficients (r^2) and limits of detection (LOD) and quantification (LOQ) in µg L^{-1} (n=5)

Compounds	Matrix	LOD	LOQ	Instrument linearity		Added (µg L^{-1})	Recovery[b]±RSD (%)
				Standards [a] concentrations range	r^2		
BaP	bile	0.83	2.50	2.5-50	0.999	5.0	96.8 ± 4.6
	plasma	0.33	1.00	2.5-50	0.999	5.0	89.9 ± 6.0
3-OH-BaP	bile	0.17	0.50	0.5-50	0.999	1.0	98.1 ± 5.5
	plasma	0.07	0.20	0.5-50	0.999	1.0	59.3 ± 6.0
						10	60.4 ± 6.8

[a] (n=12; 7 levels in µg L^{-1} methanol in duplicate).

[b] (n=5 determinations)

3.3.8. Chromatographic conditions

The liquid chromatographic system used was a Hitachi LaChrom Elite HPLC, which was constituted of an L-2130 quaternary pump and an in line degasser, a L-2485 FL detector (FD), and a L-2200 autosampler. Separations were performed with a 250 x 4.6 mm (length × i.d.), 5 µm particle, Purospher® STAR RP-18e analytical column obtained from Merck (Germany) and a 4 × 4 Chmm i.d., 5 µm particle, guard column with the same packing material.

Mobile phases used, methanol (A) and water (B), were at a flow rate of 1 mL min^{-1}. The injection volume was set to 50 µL. For BaP elution, the temperature of the column was maintained at 33 °C, and the following gradient was used: 95% A change to 100% A in 5 min, hold for 10 min, change to 95% A in 1 min and, finally, hold for 10 min giving an analysis time of 26 min. For 3-OH-BaP elution, the temperature of the HPLC column was kept constant at 40 ºC and the gradient was: 70% A for 5 min, change to 95% A in 1 min, hold for 10 min, change to 70% A in 1 min and, finally, hold for 10 min. Total run time was 27 min per sample. The excitation and emission wavelengths for BaP and 3-OH-BaP detection, were 296/406 and 308/432, respectively (Rey-Salgueiro et al. 2008).

3.3.9. Statistical analysis

Exposure time and treatment effects were evaluated by means of a one-way ANOVA for each one of the mentioned factors, followed by a multiple comparision test (Tukey's test) at a 5% significance level. Some data had to be log or sqrt transformed in order to fit ANOVA assumptions. Correlation (=r) between all the parameters measured was tested by Pearson correlation analysis. All the statistical tests were performed using the software Statistica 7 (Statsoft, Inc., 2004).

3.3.10. Ethics statement

The animals used in the research described in this paper were treated in accordance with the Portuguese Animals and Welfare Law (Decreto-Lei nº 197/96) approved by the Portuguese Parliament in 1996.

3.4. Results

3.4.1. BaP concentration in water

In order to know the real concentration of BaP in the water of experimental aquaria, BaP levels were measured at different times after the addition at 0 d and 14 d of the experiment. No differences were observed between day 0 and 14 in any of the collection periods, so the results presented were combined (figure 3.1). There was a decline of BaP concentration over time at both concentrations and no BaP was detected in the solvent control group. Eight hours after the addition of BaP stock solution there was a marked decrease in BaP, mainly at the lower concentration (88%) while for 100 µg L^{-1} it was 41%. The decrease in BaP concentration was even more significant, for both concentrations, 24 hours after the addition disappearing almost completely at lower concentration.

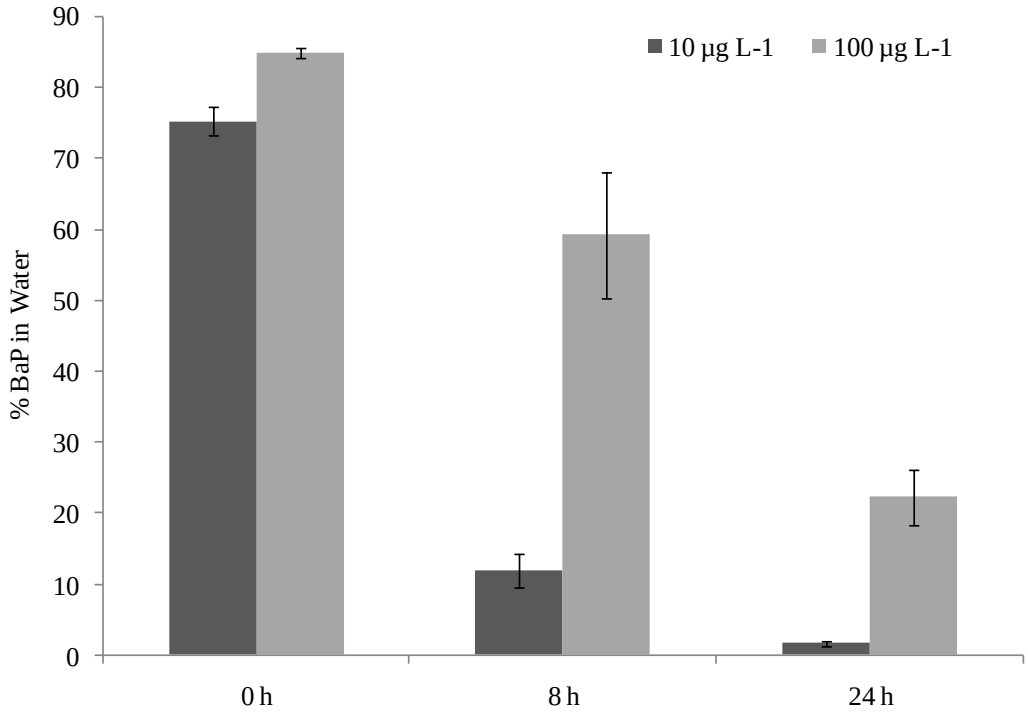

Figure 3.1 - BaP (in percentage) in water at 0, 8 and 24 h after the addition of 10 and 100 µg of BaP L^{-1} of water.

Values are presented as mean ± standard error.

3.4.2. Bile and plasma analysis

BaP was not found in bile and plasma of non-exposed and exposed animals. The metabolite, 3-OH-BaP, was detected in free and conjugated forms in bile of exposed animals as can be seen in figure 3.2. The metabolite was not found in bile of solvent control animals, neither as free nor as conjugated form. Free 3-OH-BaP represented 0.04 ± 0.01% and 0.03 ± 0.01% of the T-3-OH-BaP for 10 and 100 µg L^{-1} treatments (figure 3.2a), respectively; thus conjugated metabolites were the major forms of 3-OH-BaP in bile, being the G-3-OH-BaP the main conjugated form present (figure 3.2c). An increase in the BaP dose in water resulted in a significant increase in free 3-OH-BaP, T-3-OH-BaP and G-3-OH-BaP levels in bile at both concentrations (figure 3.2). Regarding the effect of exposure time, a significant increase in free 3-OH-BaP after 14 d of exposure to higher BaP concentration was detected in comparison to 7 d. However, no significant differences were observed between days within the same treatment for T-3-OH-BaP and G-3-OH-BaP.

In plasma, only one pooled sample contained measurable levels of free 3-OH-BaP (0.40 ng mL^{-1}) from the longer exposure period to the higher BaP exposure levels. T-3-OH-BaP concentration in animals exposed to 10 µg L^{-1} was near the detection limit and the highest levels were quantified in fish exposed to 100 µg L^{-1} (figure 3.3). In contrast to bile, there was a significant increase of T-3-OH-BaP 14 d after exposure to higher BaP concentration in comparison to 7 d of exposure.

The levels of free 3-OH-BaP ($r=0.73$; $p<0.05$), T-3-OH-BaP ($r=0.89$; $p<0.05$) and G-3-OH-BaP ($r=0.88$; $p<0.05$) in bile were correlated with T-3-OH-BaP levels measured in plasma.

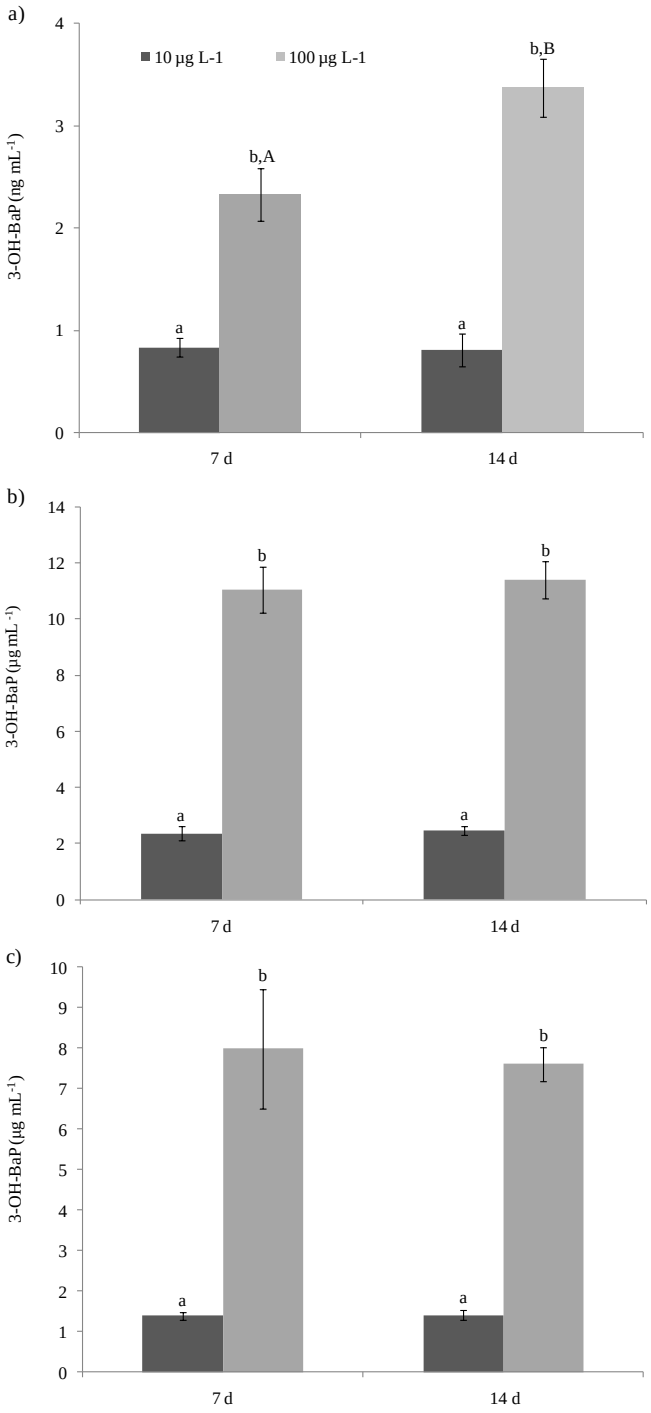

Figure 3.2 - Free (a), total (b) and glucuronide (c) 3-OH-BaP levels in bile after waterborne exposure to BaP (10 and 100 µg L^{-1}) for 7 and 14 d.

Values are shown as mean ± standard error. Different lowercase letters denote significant differences ($p<0.05$) between groups within the same day of exposure. Different capital letters denote significant differences ($p<0.05$) between days within the same treatment.

3.4.3. UGT activities and correlations between biotransformation enzymes and 3-OH-BaP levels

UGT had mean activities in control groups of 833.35 ± 37.90, 528.36 ± 72.78 and 794.52 ± 92.62 pmol min^{-1} mg^{-1} protein, in liver, intestine and gill, respectively. UGT activity in exposed animals was not different from solvent control in any of tissues analyzed, however a significant increase in UGT activity was seen from 7 to 14 d at 10 µg L^{-1} in intestine and gill. UGT activity at 100 µg L^{-1} exposure was significant lower than at 10 µg L^{-1} at 14 d in intestine and gill (table 3.2).

Nile tilapia EROD and GST activities in liver, intestine and gills after waterborne exposure to BaP (10 and 100 µg L^{-1}; a previous work developed in our laboratory) (Costa et al. 2011) and UGT activities measured in this study are presented in table 3.2. Positive statistically significant correlations ($p<0.05$) were observed between T-3-OH-BaP concentrations in plasma and EROD activities in liver ($r=0.51$), intestine ($r=0.73$) and gills ($r=0.76$). The same was also observed between free 3-OH-BaP level in bile and EROD activities in intestine ($r=0.71$; $p<0.05$) and gill ($r=0.59$; $p<0.05$), in contrast, the free metabolite was not correlated with this enzyme activity in liver. Positive statistically significant correlations were obtained between T-3-OH-BaP concentration, in plasma and in bile, and GST activity in liver ($r=0.82$ and $r=0.68$, respectively; $p<0.05$) but, were not correlated with activity in intestine and gill.

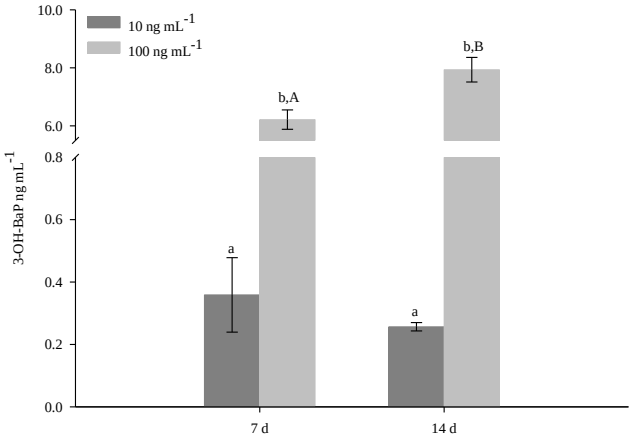

Figure 3.3 - Total 3-OH-BaP levels in plasma after waterborne exposure to BaP (10 and 100 µg L^{-1}) for 7 and 14 d.

Values are shown as mean ± standard error. Different lowercase letters denote significant differences ($p<0.05$) between groups within the same day of exposure. Different capital letters denote significant differences ($p<0.05$) between days within the same treatment.

Table 3.2 - EROD, GST and UGT activities in liver, intestine and gill of Nile tilapia after waterborne exposure to BaP (10 and 100 µg L^{-1})

Values are expressed in percent of activity (pmol min^{-1} mg^{-1} protein for EROD and UGT; nmol min^{-1} mg^{-1} protein for GST) in relation to solvent control (set to 100%), and are shown as mean ± standard error

Tissue	BaP dose (µg L^{-1})	EROD *		GST *		UGT	
		7 d	14 d	7 d	14 d	7 d	14 d
Liver	10	310.49 ± 57.17 #	222.59 ± 37.75	93.82 ± 5.17	90.64 ± 6.44	99.35 ± 12.98	83.23 ± 10.19
	100	744.44 ± 144.38 #	504.91 ± 96.80 #	113.14 ± 12.98	139.20 ± 5.71 #	106.73 ± 13.61	74.70 ± 13.98
Intestine	10	510.15 ± 248.45	624.32 ± 183.13	77.90 ± 29.01	62.48 ± 5.29	73.37 ± 14.75 A	124.15 ± 18.48 B, a
	100	1586.73 ± 790.71 #	2372.01 ± 307.26 #	68.97 ± 14.43	35.81 ± 2.83 #	81.96 ± 17.25	73.37 ± 5.14 b
Gill	10	643.74 ± 29.77 #	799.58 ± 91.49 #	97.99 ± 9.57	119.56 ± 24.14	92.64 ± 8.13 A	132.92 ± 12.98 B, a
	100	1320.66 ± 378.50 #	1738.96 ± 196.69 #	166.13 ± 14.96 #	118.21 ± 13.52	82.71 ± 10.77	79.76 ± 11.15 b

* (Costa et al., 2011)

Significant differences ($p<0.05$) in comparison to solvent control

A, B Significant differences ($p<0.05$) between days within the same treatment

a, b Significant differences ($p<0.05$) between groups within the same day of exposure

3.5. Discussion

The contamination of aquatic ecosystems by organic contaminants as PAHs is a matter of great concern in environmental toxicology. BaP is one of the most common and toxic PAHs, and its toxicity is mainly linked to the capacity of fish species to biotransform it into reactive metabolites. The present study aimed at evaluating BaP metabolism into 3-OH-BaP, a toxic metabolite, and the metabolite bioavailability in bile and plasma of Nile tilapia after waterborne exposure.

The real concentration of BaP detected in the water from the experimental aquaria was significantly lower than the nominal concentration (figure 3.1). The decrease of BaP concentration observed in water was in line with studies where the stability of BaP was evaluated in similar conditions as the ones in our study but without fish (Swietlik et al. 2002); the higher decrease of BaP observed in our experimental set up could be explained by the presence of fish. Moreover, due to the same number of animals in aquaria (at both concentrations), a higher decline of BaP was also observed at the lower concentration.

Fish have a high capacity to metabolize PAHs, thus no BaP was detected in bile and plasma of Nile tilapia and only the detection of BaP metabolites (free and conjugated). Hence, our results show the uptake and the ability of Nile tilapia to metabolize BaP and neutralize the toxic metabolite, showing to possess a well developed biotransformation system, eliminating the most toxic forms of BaP (free 3-OH-BaP) into non toxic forms (conjugated form). In bile, the predominance of conjugates over free metabolites, mainly G-3-OH-BaP, was consistent with results observed in *Fundulus heteroclitus* after waterborne exposure at the same concentrations of BaP (Zhu et al. 2008). In different exposure conditions, by single intraperitoneal injection (10 mg Kg^{-1}), Ictalurid catfish bile showed to have also a higher percentage of billiary G-3-OH-BaP (Willett et al. 2000). Contrary to the observed dose dependent response, time dependence responses were observed only with free 3-OH-BaP in bile. Comparable results, upon waterborne exposure to BaP, have been reported in *Sparus macocephalus* (Wang et al. 2008) and in sole (*Solea solea*), after dietary exposure to an equimolar mixture of BaP, pyrene and fluoranthene (Wessel et al. 2010). In contrast, when measuring total BaP metabolites as fluorescent aromatic compounds (FACs) in bile by fixed wavelength fluorescence, instead of the HPLC analysis, as in the previous studies (including this one), significant time dependent responses after BaP waterborne exposure were reported (Boleas et al. 1998; Costa et al. 2011). The assessment of FACs in bile gives the total metabolites and not specific ones, so analytical techniques, like HPLC, that allow measurement of individual

compounds, give more precise information about PAHs metabolism, especially when it concern to free metabolites and conjugation.

In plasma, free 3-OH-BaP was detected in some plasma samples, but quantification was only possible in one pooled sample of animals exposed to higher BaP concentration for 14 d (0.40 ng mL^{-1}). The low concentrations of free 3-OH-BaP detected in plasma could be due to it propensity to bound with components of the blood and form unextractable products (James et al. 2001), or due to the rapid phase II conjugation in liver and extrahepatic tissues (James et al. 1996). T-3-OH-BaP metabolite was detected and the levels were considerably lower than the ones detected in bile (0.30 ± 0.04 ng mL^{-1} vs. 2.50 ± 0.10 µg mL^{-1} in 10 µg L^{-1} at 14 d).

In the present work, despite the high G-3-OH-BaP levels detected in bile no induction of UGT was observed in liver, intestine or gill. Even though an increase in UGT activity over time was verified at lower BaP exposure level in intestine and gill. The lack of UGT induction by BaP in Nile tilapia in the present study is in agreement with the low sensitivity of this enzyme that has been reported in fish collected in field (Della Torre e al. 2010; Schereiber et al. 2006). Moreover after exposure via intraperitoneal injection (10 and 50 mg Kg^{-1}), similar results were showed in longear sunfish (Lepomis megalottis) (Brammell et al. 2010). In a previous study, EROD and GST induction activities were shown in liver, intestine and gill of Nile tilapia after BaP exposure under similar experimental conditions (Costa et al. 2011). A joint analysis of the results obtained in this study and those previously published, showed positive correlations between enzymatic activities and free and T-3-OH-BaP levels in bile and plasma. The gill and intestine EROD induction (Costa et al. 2011) and the positive correlations between enzymatic activities and T-3-OH-BaP concentration in plasma and free 3-OH-BaP in bile, strengthen the idea that these extrahepatic tissues are involved in the metabolism of BaP and that the metabolites formed are released into the blood stream. Moreover, phase II enzymes activity (GST and UGT) in intestine and gill and the predominance of conjugates metabolites in plasma, corroborate that these extrahepatic tissues play also an important role in the elimination of the toxic form of 3-OH-BaP. Taken together these results indicate that liver and extrahepatic tissues as intestine and gill can be important sources of metabolites into the blood but in addition, they work together to reduce the circulating levels of free 3-OH-BaP.

The major concern about exposure to BaP is the toxic effects produced by the presence of the toxic form of 3-OH-BaP. Some studies have evaluated the in vitro 3-OH-BaP effects and showed that low concentrations of 3-OH-BaP (from 8 to 67 ng mL^{-1}) can have estrogenic activity (van Lipzig et al. 2005) and form covalent bindings with

macromolecules (Moorthy et al. 2003; Sugihara and James 2003), leading to toxic effects at the organism level. Even though we have not evaluated toxic effects and the levels of free 3-OH-BaP measured in plasma (0.40 ng mL^{-1}) were lower than concentrations capable of producing toxic effects, since it was quantified in one pooled sample, the levels obtained in our study could be diluted. Hence, individual fish can have higher concentrations than the ones determined and as a consequence being exposed to toxic effects of this reactive form of 3-OH-BaP.

3.6. Conclusion

This study has shown that the capacity of Nile tilapia to metabolize BaP resulted in high concentrations of metabolites in bile and plasma. The predominance of conjugated over free forms of 3-OH-BaP in bile and plasma indicated the importance of phase II in metabolism and elimination of BaP in Nile tilapia. Our data suggest extrahepatic metabolism of BaP and as responsible for the presence of 3-OH-BaP metabolite in plasma. Although low levels of free 3-OH-BaP, reactive form, were detected in plasma, we must not exclude possible toxic effects in Nile tilapia.

3.7. Acknowledgements

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Chapter 4

Gene expression analysis of ABC efflux transporters, CYP1A and GSTα in Nile tilapia after exposure to Benzo(a)pyrene

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4. Gene expression analysis of ABC efflux transporters, CYP1A and GSTα in Nile tilapia after exposure to Benzo(a)pyrene

4.1. Abstract

The aim of this study was to evaluate the response of ABC transporters, CYP1A and class alpha (α) GST genes, upon water and dietary exposures to benzo(a)pyrene (BaP) in *Oreochromis niloticus*. Partial mRNA sequences of ABC transporters (*ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2* and *ABCG2*) were identified, and their tissue distribution patterns evaluated in liver, gill and intestine, showing similarities with other fish and mammals. After 14 days of water exposure to BaP, ABC transporters mRNA expression was up-regulated, namely *ABCC2* in gill (up to 16-fold) and *ABCG2* in liver (up to 2-fold) and proximal intestine (up to 7-fold). *CYP1A* mRNA expression was up-regulated in water exposed animals, with maximum fold inductions of 5, 35 and 155, respectively in liver, gill and proximal intestine. After dietary exposure, intestinal *CYP1A* mRNA showed a 13-fold increase in exposed animals. No significant changes were seen in *ABCB1b*, *ABCC1* and *GSTα* mRNA expression after both routes of exposure to BaP. In conclusion, this study has shown that transcriptional expression of some ABC transporters and *CYP1A* respond to the presence of BaP, indicating a possible involvement and cooperation in the detoxification process in Nile tilapia.

Keywords: *ABC transporters, Cytochrome P450, Glutathione S-transferase alpha Detoxification, Fish, Oreochromis niloticus*

4.2. Introduction

The ATP binding cassette (ABC) superfamily is a highly conserved family of transmembrane proteins (Dean and Annilo, 2005), and some of its members confer Multixenobiotic Resistance (MXR) in aquatic organisms by acting as efflux pumps of a wide variety of putative toxicants, and/or its metabolites (Kurelec, 1992). MXR is similar to a previously described phenotype of Multi-Drug Resistance (MDR), first observed in mammalian tumor cells (Gottesman et al., 1996), and related to the over expression of an ABC transporter (ABCB1) responsible for the efflux of a high number of anti-cancer agents (Chan et al., 2004). In addition to ABCB1 (P-glycoprotein, Pgp), studies using various animal models, have indicated ABCCs 1-5 (Multiresistance Associated Proteins, MRPs) and ABCG2 (Breast Cancer Resistance Associated Protein, BCRP) as the most relevant in the toxicological context (reviewed in Leslie et al., 2005). Another transporter from the ABCB family, ABCB11 (Bile Salt Export Pump, BSEP), has also been studied in aquatic organisms (Zaja et al., 2008a; Zaja et al., 2008b; Loncar et al., 2010) mostly due to its high degree of similarity with ABCB1, since mammalian studies indicate that ABCB11 exports bile salts from hepatocytes into bile canaliculus, and is not involved in the efflux of xenobiotics (Gerloff et al., 1998). In aquatic toxicology, research on these transporters has been mainly focused on ABCB1 (Kurelec, 1992; Bard et al., 2002a; Bard et al., 2002b) while the remaining ABC transporters are less studied. Although they have been identified in different fish species (Zaja et al., 2008a; Zaja et al., 2008c; Paetzold et al., 2009; Fischer et al., 2010; Loncar et al., 2010) both at gene and protein levels, only a few studies have investigated the modulation of these transporters by environmental pollutants in fish (Bard et al., 2002a; Bard et al., 2002b; Paetzold et al., 2009; Zucchi et al., 2010; Long et al., 2011a; Long et al., 2011b).

These proteins translocate a wide variety of substances and, although there is some overlap in substrate specificity, there are differences in transport mechanisms and chemical composition of substrates. ABCB1 transports moderately hydrophobic, amphiphilic, neutral or positively charged substances, including exogenous and endogenous compounds, while ABCCs and ABCG2 primarily transport organic anions conjugated to glutathione, glucoronide, sulphate or other polar groups (Leslie et al., 2005). Considering these characteristics, it has been previously suggested that ABC transporters may be similarly regulated in a coordinated fashion with phase I and II enzymes, providing an important mean of protection to the cell from xenobiotic insults (Bard, 2000; Leslie et al., 2005; Xu et al., 2005). It is believed that ABCB1 acts as first

line of defense preventing unmodified compounds from accumulating in the cell (phase 0 of cellular detoxification), while ABCCs and ABCG2 transport phase I and II metabolites, therefore acting in phase III of cellular detoxification (Bard, 2000; Sturm et al., 2005). Induction of phase I cytochrome P4501A (CYP1A) and phase II glutathione S-transferases (GSTs) towards many environmental pollutants has been widely studied, including to Polycyclic Aromatic Hydrocarbons (PAHs) which are known agonists of the Aryl hydrocarbon Receptor (AhR) that controls the expression of these genes (for a review see van der Oost et al., 2003). Recent studies have evaluated the hepatic expression of some ABC transporters and phase I and/or phase II enzymes in the presence of pollutants (Paetzold et al., 2009; Della Torre et al., 2010; Zucchi et al., 2010), but the exact way of action of these putatively important parts of the cellular detoxification has not been experimentally demonstrated yet. Based on substrate specificity, immunological cross reactivity and protein sequence data, mammalian citosolic GSTs have been grouped into seven classes: Alpha (α), Mu, Pi, Theta, Sigma, Omega and Zeta (Hayes et al., 2005). GSTα is one of the most expressed classes in fish (Fu and Xie 2006; Li et al., 2010) and has been associated with a multidrug-resistance phenotype observed in rats (Hayes et al., 1995). Benzo(a)pyrene (BaP) is a common PAH, widely spread in the aquatic environments, whose carcinogenic and mutagenic properties have been extensively studied (Buhler and Williams, 1989; Tsukatani et al., 2003), and was used as a model contaminant in this study. BaP is a known inducer of phase I and II enzymes, like CYP1A and GSTs, and the transport of the parent compound and/or its metabolites by ABC transporters proteins has been hypothesized (Chao Yeh et al., 1992; Buensen et al., 2002; Bard 2000; Lampen et al., 2004; Myllynen et al., 2007) and remains a matter of debate. Taking in account that in aquatic animals the uptake of pollutants can occur through water, or through contact with food or sediments, we have exposed the model specie Nile tilapia (*Oreochromis niloticus*) to BaP, by waterborne and dietary exposure routes. Besides of being an economically important cultured species, namely in Asia and Africa, Nile tilapia is also a well established model in many toxicological studies (Almeida et al. 2001; Straus 2003; Coimbra et al. 2005; Figueiredo-Fernandes et al. 2006; Coimbra et al. 2007; Costa et al. 2011; Rey-Salgueiro et al., 2011).

The main objectives of this work were 1) the identification and sequence characterization of toxicologically relevant ABC transporters (*ABCB1*, *ABCB11*, *ABCC1*, *ABCC2* and *ABCG2*) in O. niloticus, and 2) the quantification of mRNA expression of ABC transporters, *CYP1A* and *GSTα* after waterborne and dietary exposures to BaP, in tissues involved with the uptake and metabolism of pollutants, as

liver, gills and intestine, in order to evaluate, at a transcriptional level, the possible correlations between ABC transporters, *CYP1A* and *GST*. Novel information is provided regarding ABC transporters identification in *O. niloticus*, and on gene expression changes upon BaP exposure in different tissues.

4.3. Materials and methods

4.3.1. Animals

Animals used in this study were born and raised in the laboratory (CIIMAR, Porto, Portugal). The corresponding breeders stock was obtained from the Aquaculture Station of UTAD (Universidade de Trás-os-Montes, Portugal). Animals were juvenile, and therefore not sexually mature, as confirmed by macroscopic analysis of the gonads when animals were sampled. Until the start of the exposure assays, fish were kept in 60 L aquaria supplied with biological filtration. Prior to the experiments, animals were randomly distributed in the experimental aquaria (30 L), and submitted to an acclimatation period of one week. All tanks were supplied with continuous aeration to maintain dissolved oxygen near saturation. Dechlorinated tap water was used at a temperature of 20±2 ºC, with a 12h:12h (light:dark) photoperiod. Fish were fed commercial food pellets (Aquasoja, Portugal), until satiation, once a day.

4.3.2. Stock solutions of BaP and preparation of contaminated food

For the waterborne exposures, stock solutions of BaP were prepared in acetone (0.5, 1.25 and 2.5 g/L) and were administered directly to the experimental aquaria. The percentage of solvent added to the experimental aquaria was 0.002%. For the dietary exposures a stock solution of BaP with a concentration of 40 g/L was prepared. The contaminated diets were prepared by immersion of food pellets in BaP stock solutions diluted in acetone, in a proportion of 0.32 ml/g of food. For control groups, nothing was added to the food pellets, and for solvent control groups only acetone was added to the food pellets. Acetone was evaporated under forced air current for 24 hours, until the pellets were completely dry, and diets were stored at -20 ºC until further use.

4.3.3. Xenobiotic exposures

After the acclimatation period, fish were exposed to BaP either via water or via food. For the water exposure, juvenile Nile tilapia (N=20, average weight of 16.36±0.82 g, average length of 9.99±0.19 cm) were exposed to nominal water concentrations of 10, 25 and 50 µg of BaP/L for 14 days. Also, a control group and a solvent control group (acetone alone) were maintained. The tested concentrations have been found in pore waters of estuaries polluted with petrochemical products (Maskaoui et al. 2002). Waterborne exposures were conducted in semi-static conditions in 30 L aquaria. Daily, 80% of the water was renewed, followed by the addition of fresh BaP solution or solvent to each one of the treatment groups. Animals were fed to satiation every two days, with the exception of the day before sampling. Sampling was performed 14 days after the contaminant addition.

For dietary exposures, fish were maintained in 30 L aquaria in continuous water flow conditions, which assured 100% of water renewal per day. Juvenile tilapia (N=16, average weight of 10.88±0.52 g, average length of 8.53±0.16 cm) were exposed to 100 and 200 µg of BaP/g of food for 14 days. A control (uncontaminated food) and a solvent control (acetone alone) were maintained. Fish were fed daily at a rate of 3% of body weight. After feeding, animals were observed, to assure that the total of the food was consumed in 2 to 3 minutes. Fish were sampled after14 days of exposure. The chosen levels of dietary exposure fall within a range of PAH concentrations that may be found in prey or in sediment that is ingested during feeding at heavily contaminated locations (Naes et al. 1995, Viguri et al. 2002).

4.3.4. BaP determination in water and food samples

To determine the real concentration of BaP in the water of the experimental aquaria, BaP was extracted from water samples according to the method described by Cheikyula et al. (2008), based on liquid-liquid extraction with n-hexane followed by HPLC analysis, under the conditions described in Rey-Salgueiro et al. (2008). After the addition of the contaminant, the real concentrations of BaP in water samples were 10.49, 22.51 and 41.46 µg/L, respectively for the nominal concentrations of 10, 25 and

50 µg/L. After 24 hours, and before the addition of fresh contaminant to the aquaria, the amount of BaP still present in each one of the treatment groups was 0.34, 0.69, 12.19 and 25.70 µg/L. In water collected from control and solvent control groups no BaP was detected at both times. Food pellets supplied to the fish in dietary exposures were also analyzed in terms of BaP concentration, according to the method described by Rey-Salgueiro et al. (2009), based on ultrasound-assisted solvent extraction, followed by HPLC analysis. The real BaP concentrations in the food pellets were 0.06, 0.07, 36.24 and 92.70 µg/g, respectively for control, solvent, 100 and 200 µg/g. When referring to the concentrations of BaP used in water and dietary treatments nominal concentrations are mentioned throughout the manuscript.

4.3.5. Sampling

Fish were anesthetized on ice cold water and sacrificed by decapitation. Liver, gills and proximal intestine (first one third of the intestine) were excised from the animal, placed in RNA later at 4 ºC overnight, and stored at -80 ºC until further use.

4.3.6. RNA isolation, RT-PCR, cloning and sequence analysis

Primer pairs for *ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2* and *ABCG2* genes were designed based on highly conserved regions among mammalian and fish species (table 4.1). The oligonucleotides were purchased from Stabvida (Portugal). Total RNA from liver, gills and proximal intestine was isolated from, approximately 20 mg of tissue, using the Illustra RNAspin Mini (GE Healthcare), according to manufacturer´s protocol. Total RNA was quantified through the measure of the optical density at λ260 nm, and its quality was assessed by separation in 1% agarose gel electrophoresis (in Tris-acetate-EDTA – TAE – buffer) stained with ethidium bromide, and by measurement of the ratio of the optical density at λ260/280 nm (1.8-2.0). One microgram of total RNA was reverse transcribed to produce the first strain of cDNA (iScript cDNA Synthesis Kit, BioRad), according to the manufacturer´s instructions. PCR was performed in a Biometra Thermocycler with 100 ng of cDNA (composed by a mixture of cDNA from liver, gill and proximal intestine in equal amounts), 0.2 mM of DNTPs, 0.4 µM of each primer and 2.5 mM of MgCl2 in a total volume of 50 µl, using Taq DNA Polymerase (5-

Prime, Inc, EUA), with the following conditions: 2 minutes denaturation at 94 ºC, 40 cycles of denaturation for 30 seconds, 30 seconds of annealing (different annealing temperatures – table 4.1) and 1 minute of 72 ºC of polymerization, and 10 minutes of final extension at 72 ºC. The aliquots of the PCR reactions were separated by gel-electrophoresis on a 2% agarose gel in TAE buffer stained with ethidium bromide, and the PCR products were visualized under UV light. The bands of expected size were excised, and eluted from the gel according to manufacturer´s protocol, using the commercial kit Illustra GFX Tm PCR DNA and Gel Band Purification Kit, GE Healthcare. The isolated fragments were then inserted in pGEM plasmid vector (pGEM® - T Easy Vector Systems – Promega) and incorporated in E. coli using Nova Blue Competent Cells (Novagen). After the selection of the correct colonies developed for 10 hours in solid medium (35 g/L LB Broth, ampycilin 0.1 mg/ml, IPTG 0.1 mM and X-Gal 100 mM) at 37 ºC, the plasmids were isolated from 5 ml of overnight culture (at 37 ºC) using Wizard Plus SV Minipreps DNA Purification System (Promega). The inserts were sequenced by Stabvida (Portugal), and the identities of all sequences were checked using the Basic Local Alignment Search Tool (Blast) at National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Predicted topologies for the aminoacid partial identified sequences were performed with the Polyphobius algorithm (http://phobius.sbc.su.se/poly.html). Alignments of the obtained sequences with other mammal and fish species were conducted with ClustalW Multiple Alignment (Thompson et al., 1994). Phylogenetic analysis was performed with Mega 4.0.2 (Tamura et al., 2007) using neighbor-joining method and a percentage of concordance based on 1000 bootstrap iterations.

Table 4.1 – Primer sequences, sequence lengths, annealing temperatures, GenBank accession numbers and homology percentages with other animal species of ABC transporters identified in Nile tilapia.

Target gene	Sense	Antisense	Sequence length (bp)	Annealing temperature	Acession Number	Homology with other species
ABCB1b	cctgtgtcgttcctcaaggt	cctctcttgtgaggcagac	922	56ºC	GQ911571	*Poeciliopsis lúcida* – 80% ; *Oncorhynchus mykiss* – 75%; *Trematomus bernachii* – 75%; *Homo sapiens* – 63%
ABCB11	catttcagcctggtgttcag	gtcagagttctggatggtgg	820	60ºC	GQ911570	*Platichthys flesus* – 93%; *Fundulus heteroclitus* – 93%; *Oncorhynchus mykiss* – 91%; *Danio rerio* – 91%; *Homo sapiens* – 84%
ABCC1	atgttcatgggctccatgttca	ggatccaggttcatcctcaga	1062	56ºC	GQ911567	*Danio rerio* – 84%; *Oncorhynchus mykiss* – 66%; *Homo sapiens* - 75%
ABCC2	ctcagtgtatctgcagtacct	ctaaacaggcagttggtaagac	1134	56ºC	GQ911569	*Oncorhynchus mykiss* – 88%; *Danio rerio* – 86%; *Monodelphis domestica* – 71%; *Homo sapiens* – 72%
ABCG2	atcggactcaatggcatcatga	gatgaagagttcagctgctga	1100	56ºC	GQ911568	*Oncorhynchus mykiss* – 90%; *Danio rerio* 88%; *Salmo salar* – 85%; *Homo sapiens* – 73%

4.3.7. Quantitative real-time PCR (qRT-PCR)

Gene expression quantification of *ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2*, *ABCG2*, *CYP1A*, *GSTα* was performed in liver, gill and proximal intestine, by means of quantitative real time PCR (qRT-PCR). Elongation factor 1 (*EF1*) and 18S ribosomal RNA (*18S rRNA*) were both evaluated as possible housekeeping genes, and *18S rRNA* was chosen since its expression proved not to be affected by the treatments, and to be stable between the analyzed tissues (data not shown). For qRT-PCR, 1 µg of total RNA was subjected to digestion of genomic DNA using Deoxyribonuclease I, Amplification Grade (Invitrogen) and first strand cDNA was synthesized using the iScript cDNA Synthesis Kit from BioRad. Specific primers for *ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2* and *ABCG2* were designed based on the previously partially sequenced genes in Nile tilapia, using Beacon DesignerTM software. Primers for *CYP1A*, *GSTα*, *18S rRNA* and *EF1* were designed based on partial mRNA sequences of *O. niloticus*, available on Genbank (accession numbers GI13365614 for *CYP1A*, EU234530 for *GSTα*, DQ397879 for *18S rRNA* and AB075952 for *EF1*), using the Primer-Blast software (http://www.ncbi.nlm.nih.gov). To confirm the identities of the amplicons for qRT-PCR, RT-PCR reactions were conducted and, after excision from the gel, products were cloned and sequenced (as described in section 4.3.6). Optimal primers concentrations were determined (600/600 nM) after evaluation of the highest fluorescence signal at lower Ct number. Primer sequences and amplicon lengths of target gene sequences are given in table 4.2. Real-time PCR amplification was done in a IQ5 Bio Rad, with the use of IQ Sybr Green Supermix (Bio Rad), with 10 µl SYBR Green mix, 2 µl of each primer (6 µM) and 1 µl of cDNA in a total volume of 20 µl. Reactions were conducted under the following conditions: 95 ºC for 3 minutes, followed by 40 cycles at 95 ºC for 10 seconds, 54 ºC for 30 seconds and 72 ºC for 30 seconds. At the end of each run a melting curve analysis was done (from 55 ºC to 95 ºC) to determine the formation of specific products. Samples were run in duplicate. No template controls were run to exclude contamination and the formation of primer dimers. To determine the efficiency of the PCR reactions (table 4.2), standard curves were made for all the genes, with 6 serial dilutions of the template (concentrations range from 0.05 to 50 ng/µl), and the slopes and regression curves were calculated. Quantification of the expression of the genes in study, *ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2*, *ABCG2, CYP1A* and *GSTα*, after exposures to BaP, was done by normalization against the housekeeping gene (*18S rRNA*). The ΔCt values were calibrated against the control ΔCt, and the relative expression of the target genes was calculated by the $2^{-\Delta\Delta Ct}$ method, considering efficiencies close to 100% (Livak and Schmittgen, 2001). For the purpose of inter-gene

comparison throughout one tissue, relative quantification was used as a method of choice. Target genes were normalized to the housekeeping gene (*18S rRNA*), according to the equation, MNE = $(E_{ref})^{Ct(ref,mean)}$ / $(E_{target})^{Ct(target,mean)}$, described in detail by Simon (2003), where MNE stands for mean normalized expression; E_{ref} is the efficiency of housekeeping gene; E_{target} is target gene efficiency; $Ct_{ref,mean}$ is the mean CT value of the housekeeping gene; and $Ct_{target,mean}$ stands for the mean Ct value of the target gene. Tissues from control animals were used, and results were presented as target gene expression relative to housekeeping gene expression multiplied by 10000.

Table 4.2 – Primer sequences, amplicon lengths and efficiency of reaction, used in ABC transporters, CYP1A, GSTα and 18S rRNA gene expression quantification by qRT-PCR in Nile tilapia

Target gene	Sense	Antisense	Amplicon length	Efficiency of the PCR reaction
ABCB1b	cgttcctcaaggtgatggct	ggctgcattgcaccattgat	91 pb	98.5%
ABCB11	ctggtcagacactggccttt	caggaaagacacgttgacgc	143 pb	110.0%
ABCC1	atccgtgagagtgaccag	caaatgacacaatgaagtttcc	117 pb	99.7%
ABCC2	cctggttggcttgtctatatcc	ctcgctgtattcactcactctc	123 pb	107.6%
ABCG2a	tcatgaagccgggtctcaac	agacctgcagggtcctttct	96 pb	103.9%
CYP1A	cgtcgtcgtctctgttgcc	catcgtcgtggtggtcatagc	70 pb	96.6%
GSTα	aaatggatggcatgaagctc	tcgttctttgggatcctttg	92 pb	109.8%
18S rRNA	cggaaggatcattactggctacac	agaccctcggcggcaaag	78 pb	100.1%

4.3.8. Statistical analysis

Treatment effects were evaluated by means of a one-way ANOVA, followed by a multiple comparision test (Tukeys test) at a 5% significance level. Some data had to be log or square root transformed in order to fit ANOVA assumptions. All tests were performed using the software Statistica 7 (Statsoft, Inc., 2001).

4.3.9. Ethics statement

The animals used in the research that is described in this paper were treated in accordance with the Portuguese Animals and Welfare Law (Decreto-Lei nº 197/96) approved by the Portuguese Parliament in 1996. Institutional animal approval by CIIMAR/UP and DGV was granted for this study.

4.4. Results

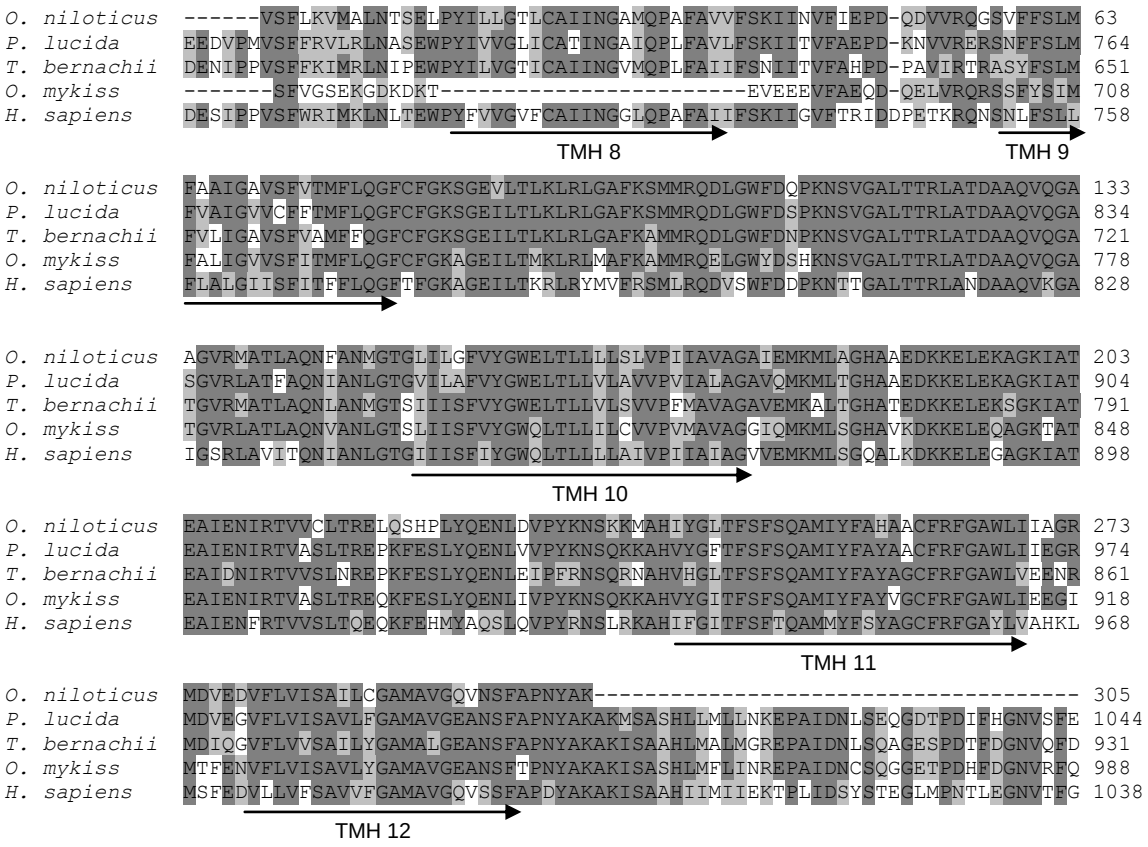
4.4.1. Identification of ABC transporters related genes

Based on the degrees of homology with the same genes in mammals and other fish species, we were able to identify partial gene sequences for *ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2* and *ABCG2* in Nile tilapia.

A 922bp sequence similar to *ABCB1b*, a 816bp sequence similar to *ABCB11*, a 804bp sequence similar to *ABCC1*, a 805bp sequence similar to *ABCC2* and a 659bp sequence similar to *ABCG2* were identified. Sequences were submitted to GenBank and given the accession numbers provided in table 4.1. Analysis performed using blastx alignments, showed high degrees of homology of the identified transcripts with other fish and mammal species, including human (table 4.1). Multiple alignments of the deduced amino acid (aa) sequences in Nile tilapia with other fish species and human are shown in figure 4.1. In the partial aa sequences of *ABCB11* and *ABCG2* the following highly conserved regions were identified: Walker-A, Walker-B and C-Motif (figure 4.1b and 4.1e). Topology analysis revealed that partial aa sequence of *ABCB11* and *ABCG2* covered almost completely a nucleotide binding domain (NBD 2 for *ABCB11* –figure 4.2b, and NBD 1 for *ABCG2* – figure 4.2e). The Walker A region was also identified in the partial aa sequences of *ABCC1* (figure 4.1c) and *ABCC2* (figure 4.1d), and according to the topology predictions, the identified sequences partially covered membrane spanning domain 2 (MSD2) and NBD2, for both *ABCC1* (figure 4.2c) and *ABCC2* (figure 4.2d). No highly conserved regions were identified in the *ABCB1b* partial aa sequence (figure 4.1a), and topology analysis revealed that *ABCB1b* sequence covered almost completely the MSD2 (figure 4.2a). Results of the phylogenetic analysis revealed that sequences identified in

Nile tilapia are evolutionarily closer to the same genes in other fish species, than to mammals (figure 4.3).

a) ABCB1b multiple alignment



b**) ABCB11 multiple alignment**

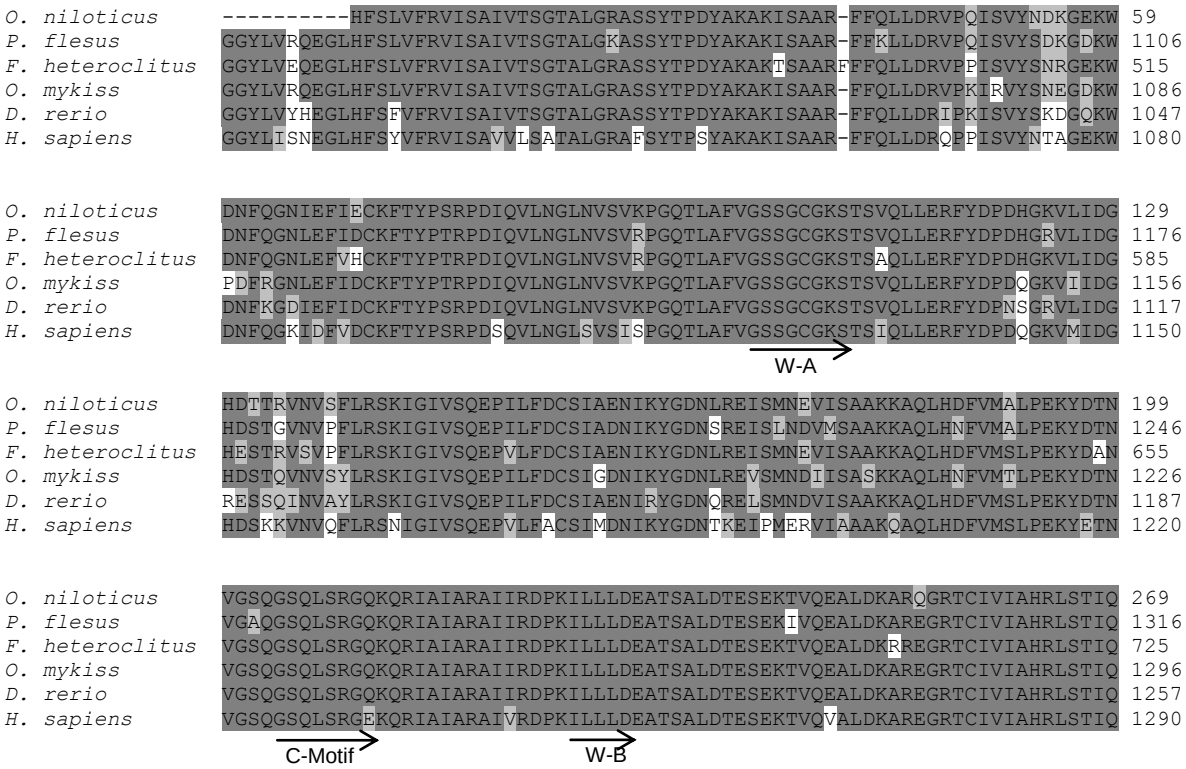


Figure 4.1

```
O. niloticus      NSD------------------------------------ 272
P. flesus         NSDIIAVMSRGYVIEKGTHNQLMLLKGAYYKLVTTGAPIS 1356
F. heteroclitus   NSDIIAVMSRGFVIEKGSHDQLMALKGAYYKLVTTGAPIS 765
O. mykiss         NSDIIAVMSRGFVIEQGPHDQLMALKGAYYKLVTTGAPIS 1336
D. rerio          NSDIIAVMSRGYVIEKGTHDYLMGLKGAYYKLVTTGAPIS 1297
H. sapiens        NADIIAVMAQGVVIEKGTHEELMAQKGAYYKLVTTGSPIS 1330
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c) ABCC1 multiple alignment

```
O.niloticus  --GLIYFFVQRFYVSSSRQLKRLESVSRSPIYTHFNETPLGTSVIRAFGEQERFIRESDQRVDHNQKAYY 68
D.rerio      PLGLLYFFVQRFYVASSRQMKRLESVSRSPVYTHFNETLLGTSVIRAFGEQQRFIKESDGRVDHNQKAYF 1187
O.mykiss     PLTLLYAFIQSFYVATSCQLRRLEAVSRSPIYTHFNETFQGASVIRAFSEQERFTLQANGRIDHNQTAYF 1124
H.sapiens    PLGLIYFFVQRFYVASSRQLKRLESVSRSPVYSHFNETLLGVSVIRAFEEQERFIHQSDLKVDENQKAYY 1075

O.niloticus  PSIVANRWLAVRLEFVGNFIVSFAALFAVIARESLSPGIMGLAISYALQLTASLTWLVRMSSDVETNIVA 138
D.rerio      PSIVANRWLAVRLEFVGNCIVTFAALFAVMARNNLSPGIMGLSISYALQVTASLNWLVRMSSELETNIVA 1257
O.mykiss     PRFVATRWLAVNLEFLGNLLVLAAATLAVMGRDTLSPGIVGLAVSHSLQVTGILSWIVRSWTDVENNIVS 1194
H.sapiens    PSIVANRWLAVRLECVGNCIVLFAALFAVISRHSLSAGLVGLSVSYSLQVTTYLNWLVRMSSEMETNIVA 1145
                                  TMH 16                TMH 17
O.niloticus  VERVKEYSDTEKEAEWKQESSSLPPGWPTKGCTDIRGFSLRYRHDLDPAIHNININGGEKVGIVGRTG 208
D.rerio      VERVKEYGDTEKEAEWKLENSNLPPGWPTAGHIEIHKFGLRYREDLELAICDISVNIAGGEKVGIVGRTG 1327
O.mykiss     VERVKEYADTPKEAPWTIEGSMLPLAWPTHGTIEMEEYGLQYRKGLDWALKGISLSIQEKEKVGIVGRTG 1264
H.sapiens    VERLKEYSETEKEAPWQIQETAPPSSWPQVGRVEFRNYCLRYREDLDFVLRHINVTINGGEKVGIVGRTG 1215
                                                                              W-A
O.niloticus  AGKSSLTLGLFRIIEAAEGHIFIDGVDIAQLGLHDLRSRITIIPQDPVLFSGSLRMNLD---------- 267
D.rerio      AGKSSLTLGLFRIIEAAEGEIRIDGVNIADLGLHELRSRITIIPQDPVLFSGSLRMNLDPFDGYTDEEVW 1397
O.mykiss     AGKSSLALGIFRILEAAKGEIYIDGINIAQIGLHELRSRITIIPQDPVLFSGSLRMNLDPFDGYSDEEVW 1334
H.sapiens    AGKSSLTLGLFRINESAEGEIIIDGINIAKIGLHDLRFKITIIPQDPVLFSGSLRMNLDPFSQYSDEEVW 1285
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d) ABCC2 multiple alignment

```
O.niloticus  NRFAKDIFTIDEAIPNSFRSWLLCFLGVLGTLFVICLATPFFAIVIIPLAVIYFFVQRFYVATSRQLRRL 70
O.mykiss     NRFAKDIFTVDEAIPQSFRSWIMCFLGVLGTLFVICLATPIFTAIIPLAVVYYFVQRFYVASSRQLRRL 1175
D.rerio      NRFAKDIFTVDEMIPMSFRSWILCLLGVLGTLFVICLATPIFTAVVVPMAVVYYFVQRFYVATSRQLRRL 1154
M.domestica  NRFANDISTVDDTIPMSFRSWTMCFLSIISTAVMICVATPVFIVVIIPLAIIYIFVQRFYMATSRQLRRL 1161
H.sapiens    NRFAGDISTVDDTLPQSLRSWITCFLGIISTLVMICMATPVFTIIVIPLGIIYVSVQMFYVSTSRQLRRL 1151
                                  TMH 15
O.niloticus  DSVSRSPIYSHFGETVSGLSVIRAYKHQDRFLKHNEVTIDENLKSVYPWIVSNRWLAIRLEFVGNLVVFF 140
O.mykiss     DSVSRSPIYSHFGETVSGLSVIRAYGHQDRFLKHNEKIIDENPKSVYLWIISNRWLAIRLEFLGNLVVFF 1245
D.rerio      DSVSRSPIYSHFGETVSGLSVIRAYGHQDRFLKHNEHTIDQNLKSVYPWIVSNRWLAMRLESLGNLVVFF 1224
M.domestica  DSVTKSPIYSHFSETVSGLSIIRAFEHQQRFLKHSFGIIDTNQKCVFSWIISNRWLAVRLELVGNLVVFF 1231
H.sapiens    DSVTRSPIYSHFSETVSGLPVIRAFEHQQRFLKHNEVRIDTNQKCVFSWITSNRWLAIRLELVGNLTVFF 1221
                                                                  TMH 16
O.niloticus  SALFAVTSRDSIDSGLVGLSISYALNVTQTLNWLVRMTSELETNIVAVERVSEYTELENEADWVTDTRPP 210
O.mykiss     LALLAVIARDSLDSGLVGLSISYALNVTQTLNWLVRMTSELETNIVAVERVSEYTEMENEADWVSGIRPS 1315
D.rerio      AALFAVISRDSLNSGLVGLSISYALNVTQTLNWLVRMTSELETNIVAVERVREYAEIQNEAPWVTSVRPP 1294
M.domestica  SALLIVIYRDNLKGDMVGLVLSNALNITQTLNWLVRMTSELETNIVSVERINEYIKVKNEAPWLLEKRPP 1301
H.sapiens    SALMMVIYRDTLSGDTVGFVLSNALNITQTLNWLVRMTSEIETNIVAVERITEYTKVENEAPWVTDKRPP 1291
                                  TMH 17
O.niloticus  QQWPEAGRVQFENYKVRYRPELDLVLHGITCDIDSTEKIGIVGRTGAGKSSLTNCLF---------- 267
O.mykiss     EKWPEAGRLRFENFKVRYRPELDLVLHGITCDIDSTEKIGIVGRTGAGKSSLTNCLFRIIEAAEGRILID 1385
D.rerio      DDWPSAGNIRFEDYKVRYRPELELVLHGVTCDIQSTEKIGIVGRTGAGKSSLTNCLFRIVEAADGRILID 1364
M.domestica  DNWPSKGEIRFTDYKVRYRPELDLILHGITCNIEGTEKIGVVGRTGAGKSSLTNCLFRILEAAEGQITID 1371
H.sapiens    PDWPSKGKIQFNNYQVRYRPELDLVLRGITCDIGSMEKIGVVGRTGAGKSSLTNCLFRILEAAGGQIIID 1361
                                                                  W-A
```

e) ABCG2 multiple alignment

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O.niloticus  ----IGLNGIMKPGLNAIMGATGSGKSSFLDVLAARKDPAGLTGEVLIDGAPQPPNFKCLSGYVVQDDVV 66
O.mykiss     KDILIDLNGLMKPGLNAIMGATGSGKSSFLDVLAARKDPAGLAGEVLMDGAPQPPNFKCLSGYVVQDDVV 131
D.rerio      KNILIGLNGIMKPGLNAILGATGSGKSSFLDVLAARKDPAGLSGEVLIDGAPQPPNFKCLSGYVVQDDVV 125
S.salar      REILVDLNGIMRPGLNAILGPTGSGKSSFLDVLAARKDPSGLSGEVLIDGAPQPPNFKCLSGYVVQDDVV 139
H.sapiens    KEILSNINGIMKPGLNAILGPTGGGKSSLLDVLAARKDPSGLSGDVLINGAPRPANFKCNSGYVVQDDVV 130
                                  W-A
O.niloticus  MGTLTVRENFTFSAALRLPSSISQKEKKAKVDRLIKELGLGRVADSRVGTQLIRGISGGERKRTNIGMEL 136
O.mykiss     LGTLTVRENFRFSAALRLPSSVSQKEKEDKVNRLITELGLTKVADSRVGTQLIRGISGGERKRTNIGMEL 201
D.rerio      MGTLTVRENLRFSAALRLPKSIKQREKDEKVERLIQELGLSKVADSRVGTQLIRGVSGGERKRTNIGMEL 195
S.salar      MGTLTVRENLRFSAALRLPRSVPQKEKEARVNDLITELGLTKVADAKVGTQMIRGISGGERKRTNIGMEL 209
```


Figure 4.1 (continued)

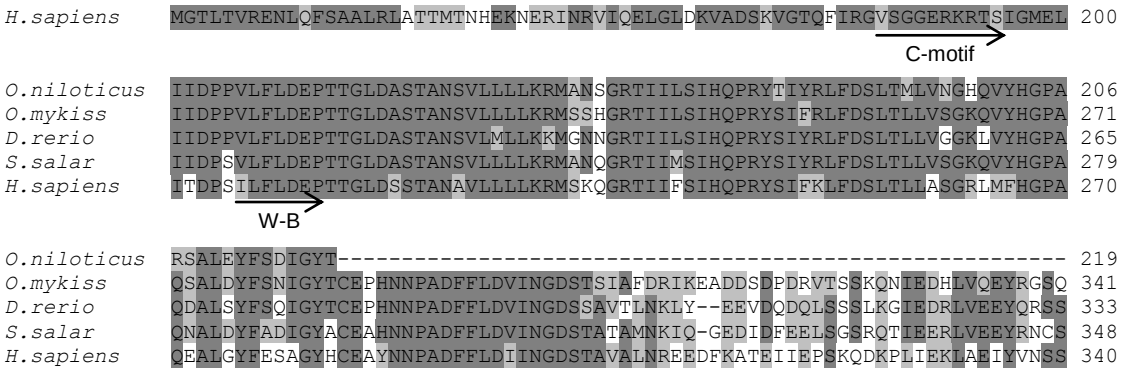

Figure 4.1 - Multiple alignments of the deduced amino acid sequences for *ABCB1b* (a), *ABCB11* (b), *ABCC1* (c), *ABCC2* (d) and *ABCG2* (e) in N. tilapia with other fish species and human.

Arrows indicate transmembrane helices (TMH), and/or ABC signature regions (C-Motif), Walker-A (W-A) and Walker-B (W-B), in each partial amino acid sequence. Identical and similar amino acids are indicated in dark and light grey, respectively. *ABCB1*: *Poeciliopsis lucida* ADQ20481.1; *Trematomus bernachii* ACX30417.1; *Oncorhynchus mykiss* AAW56424; *Homo sapiens* NP000918.2. *ABCB11*: *Platichthys flesus* CAC86593.1, *Fundulus heteroclitus* AAD29692.1, *Oncorhynchus mykiss* NP001118128.1, *Danio rerio* XP001923538, *Homo sapiens* NP003733.2. *ABCC1*: *Danio rerio* XP001341895.3, *Oncorhynchus mykiss* NP001161802.1, *Homo sapiens* NP004987.2. *ABCC2*: *Oncorhynchus mykiss* NP001118127.1, *Danio rerio* XP002664118, *Monodelphis domestica* XP001372990.1, *Homo sapiens* CAB45309.1. *ABCG2*: *Oncorhynchus mykiss* NP001118155.1, *Danio rerio* NP001036240.1, *Salmo salar* NP001167126.1, *Homo sapiens* AAH92408.1. Alignments were done with Clustal W program.

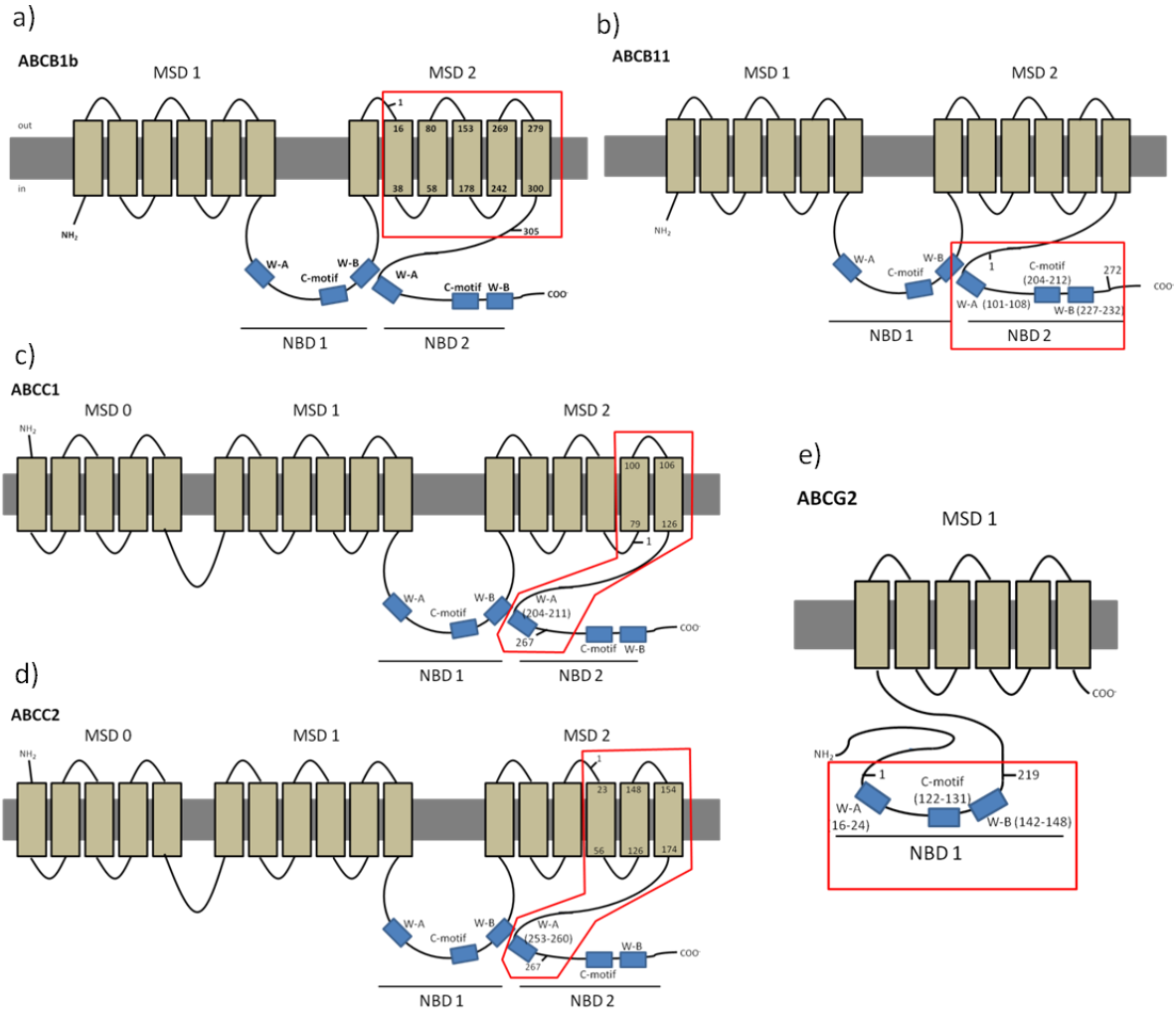

Figure 4.2 – Predicted topologies of Nile tilapia partial ABCB1b (a), ABCB11 (b), ABCC1 (c), ABCC2 (d) and ABCG2 (e) proteins with membrane spanning domains (MSDs) and nucleotide binding domains (NBDs) according to the Polyphobius algorithm.

In NBDs, highly conserved regions of the ABC transporters superfamily are indicated: W-A (Walker A), C-motif (Signature motif) and W-B (Walker B). Regions of partial ABC transporters aa sequences identified in this study are enclosed in red frame.

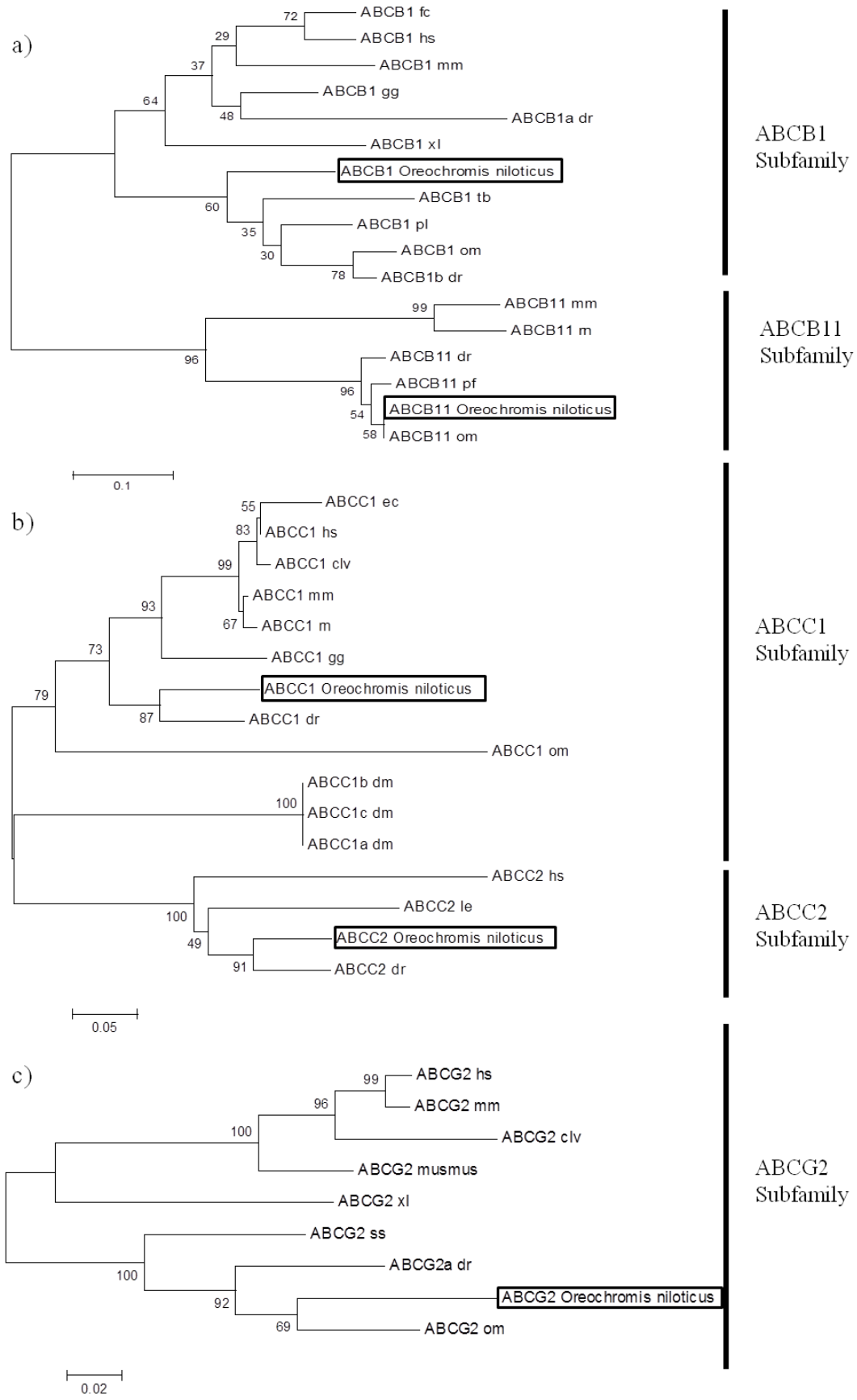
a)
ABCB1 fc
ABCB1 hs
ABCB1 mm
ABCB1 gg
ABCB1a dr
ABCB1 xl
ABCB1 Oreochromis niloticus
ABCB1 tb
ABCB1 pl
ABCB1 om
ABCB1b dr
ABCB11 mm
ABCB11 m
ABCB11 dr
ABCB11 pf
ABCB11 Oreochromis niloticus
ABCB11 om
ABCB1 Subfamily
ABCB11 Subfamily
0.1
b)
ABCC1 ec
ABCC1 hs
ABCC1 clv
ABCC1 mm
ABCC1 m
ABCC1 gg
ABCC1 Oreochromis niloticus
ABCC1 dr
ABCC1 om
ABCC1b dm
ABCC1c dm
ABCC1a dm
ABCC2 hs
ABCC2 le
ABCC2 Oreochromis niloticus
ABCC2 dr
ABCC1 Subfamily
ABCC2 Subfamily
0.05
c)
ABCG2 hs
ABCG2 mm
ABCG2 clv
ABCG2 musmus
ABCG2 xl
ABCG2 ss
ABCG2a dr
ABCG2 Oreochromis niloticus
ABCG2 om
ABCG2 Subfamily
0.02

Figure 4.3 - Phylogenetic trees based on the multiple alignments (Clustal W) of closely related proteins from ABCB, ABCC and ABCG subfamilies found in other species.

Evolutionary history was inferred using neighbor-joining method and the percentage of concordance based on 1000 bootstrap iterations is shown at the nodes. a) ABCB subfamily: ABCB1 fc – *Felix catus* NP001164535.1, hs – *Homo sapiens* NP000918.2, mm – *Mus musculus* NP035205.1, gg – *Gallus gallus* NP990225.1, dr – *Danio rerio* a ENSDARG00000021787, xl – *Xenopus laevis* NP001081394.1, tb – *Trematomus bernachii* ACX30417.1, om – *Oncorhynchus mykiss* AAW56424, dr – *Danio rerio* b ENSDARG00000010936, pl – *Poeciliopsis lucida* ADQ20481.1; ABCB11 mm – *Mus musculus* NP066302.2, rn – *Ratus norvegicus* NP113948.1, dr – *Danio rerio* XP001923538, pf – *Platichthys flesus* CAC86593.1, om – *Oncorhynchus mykiss* NP001118128.1; b) ABCC subfamily: ABCC1 ec – *Equinus caballus* NP001075232, hs – *Homo sapiens* NP004987.2, clv – *Canis lupus vulgaris* NP001002971.1, mm – *Mus musculus* NP032602, rn – *Ratus norvegicus* NP071617, gg – *Gallus gallus* NP001012540.1, dr – *Danio rerio* XP001341895.3, om – *Onchorhynchus mykiss* NP001161802.1, dm – *Drosophila melanogaster* NP723772.2-a, NP609591.2-b, NP995699.1-c; ABCC2 hs – *Homo sapiens* CAB45309.1, le – *Leucoraja erinacea* AAL92112.1, dr – *Danio rerio* XP002664118; c) ABCG subfamily: ABCG2 hs – *Homo sapiens* AAH92408.1, mm – *Macaca mulatta* NP001028091.1, clv – *Canis lupus vulgaris* NP 001041486, musmus – *Mus musculus* NP036050.1, xl – *Xenopus laevis* NP001091141, ss – *Salmo salar* NP001167126.1, dr – *Danio rerio* NP001036240.1, om – *Oncorhynchus mykiss* NP 001118155.1. Tree was designed using Mega 4.0.2 software.

4.4.2. Quantification of *ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2*, *ABCG2*, *CYP1A* and *GSTα* mRNA expression in liver, gill and proximal intestine of Nile tilapia, after BaP exposures

4.4.2.1. ABC transporters, *CYP1A* and *GSTα* mRNA expression in the different tissues

Relative quantification was used for comparison of different genes mRNA expression within each target tissue, and results are given in figure 4.4 (a-c). The highest levels of *ABCB1b* mRNA relative expression were found in proximal intestine (155-fold), while in liver expression was about 9 times lower; oppositely, *ABCB11* mRNA expression was about 8 times higher in liver (26.2-fold) than in proximal intestine (3.4-fold). Neither *ABCB1* nor *ABCB11* mRNA expression were detected in gill. *ABCC1* mRNA expression increased from liver (8.6-fold), to proximal intestine (14.1-fold) and to gill (36.9-fold). Liver and gill showed similar levels of *ABCC2* mRNA expression (15.1 and 8.9-fold, respectively) while in proximal intestine expression was about 10 times higher (104.1-fold). *ABCG2* was about 3 times more expressed in proximal intestine (23.5-fold) than in liver and gill (7.9-fold). *CYP1A* mRNA was much more expressed in liver and gill (3183.1 and 3462.4-fold, respectively), than in proximal intestine, where its expression was about 17 times lower (199.2-fold). The highest mRNA expression of *GSTα* was seen in gill (1842.0-fold). In proximal intestine and liver its expression was 20 and 100 times lower (86.0 and 19.0-fold, respectively). Considering each tissue individually, in liver, *CYP1A* mRNA expression was different ($p<0.05$) from all ABC transporters and *GSTα*, and *ABCB1b* and *ABCB11* were the most expressed transporters (figure 4.4a). In gill, *CYP1A* and *GSTα* showed a markedly higher expression than ABC transporters, and *ABCC1* was the highest expressed transporter (figure 4.4b). In proximal intestine, results showed similar expressions of *ABCB1b*, *ABCC2* and *CYP1A*, while *ABCB11*, *ABCC1*, *ABCG2* and *GSTα* mRNA expression was lower (figure 4.4c).

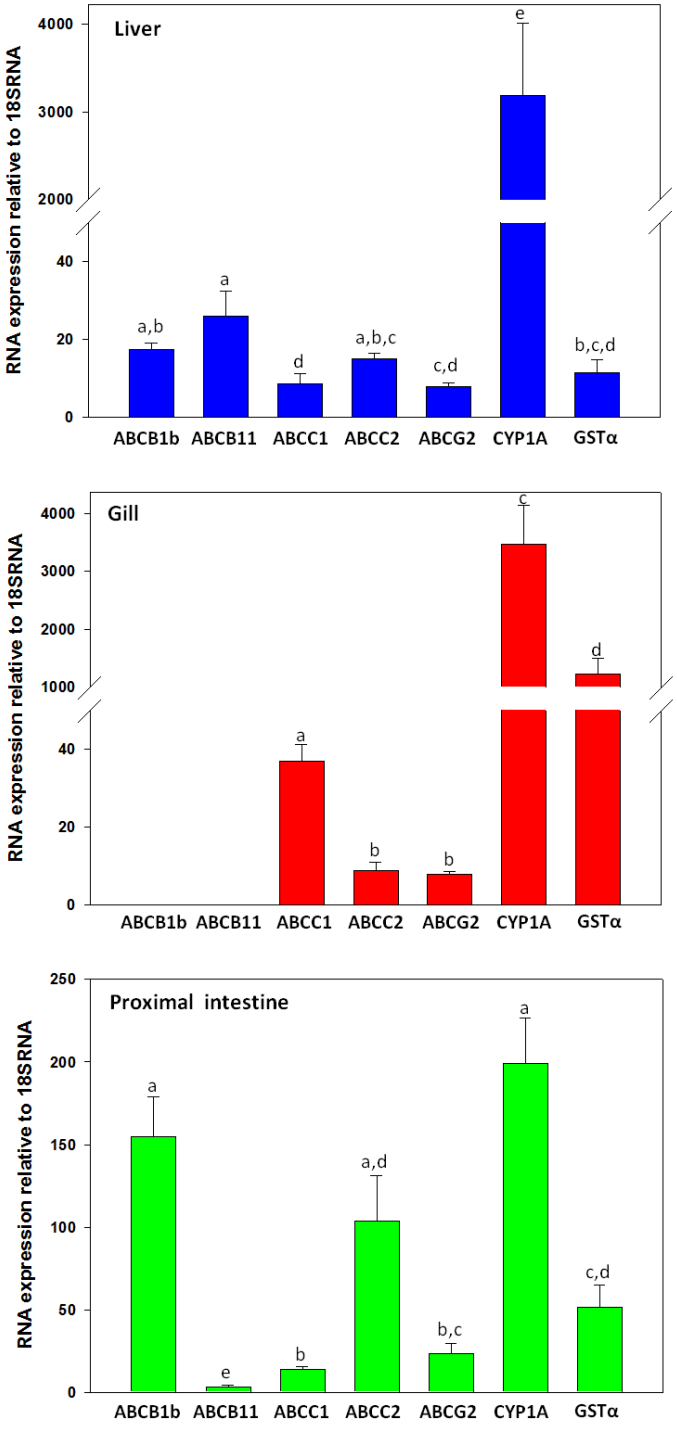

Figure 4.4 – Relative mRNA expression of ABC transporters, *CYP1A* and *GST α* in Nile tilapia liver (a), gill (b) and proximal intestine (c).

Neither *ABCB1b* nor *ABCB11* mRNA expression was detected in gill. *18S rRNA* mRNA expression was set to 10000 in all tissues. Different letters denote significant differences ($p<0.05$) between genetic expression of target genes (*ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2*, *ABCG2*, *CYP1A* and *GSTα*) within the same tissues. Results are presented as mean±SE (n=4).

4.4.3. Water exposure to BaP

ABC transporters, *CYP1A* and *GSTα* mRNA expression levels after water exposure to different BaP concentrations are displayed in figure 4.5 (a-f).

Relative mRNA expressions of *ABCC2* (in gill) and *ABCG2* (in liver and proximal intestine) were up regulated after BaP exposure, with significant differences to control animals. Gill mRNA *ABCC2* fold induction over control was dose-dependent, ranging from 6 (in 10 µg/L) to 16 (in 50 µg/L) (figure 4.5c). Similar results were seen in intestinal *ABCG2* mRNA expression, where a maximum 7-fold increase was detected in animals exposed to 50 µg/L of BaP. In liver, *ABCG2* mRNA expression had a maximum fold increase of 2 after exposure to 25 µg/L (figure 4.5d). *ABCB1b* followed a pattern of increasing mRNA expression in both liver and proximal intestine as the concentration of BaP rose (figure 4.5a), even though without statistical support. *CYP1A* mRNA was significantly up-regulated after waterborne BaP exposure in all three tissues (figure 4.5e). In liver, animals exposed to 50 µg/L of BaP showed a relative 5-fold induction in *CYP1A* mRNA expression. In gill and proximal intestine, induction of *CYP1A* mRNA was seen at all BaP concentrations, with relative increases of 14 and 19-fold (at 10 µg/L), 11 and 81-fold (at 25 µg/L) and 35 and 155-fold (at 50 µg/L), respectively for gill and proximal intestine. No significant differences were seen in *GSTα* mRNA expression in all three tissues, although an increasing pattern with BaP concentration was patent in gill (figure 4.5f).

Expression of *CYP1A* was highly correlated with *ABCC2* mRNA in gill (r=0.99, p=0.002) and with *ABCG2* mRNA expression in intestine (r=0.99, p=0.05). Both in gill and proximal intestine, high *CYP1A* correlations matched the ABC transporters that showed dose-dependent mRNA up-regulations after BaP waterborne exposure. *GSTα* and *ABCC2* mRNA expressions were highly correlated (r=0.97, p=0.004) in gill tissue. Biliary BaP metabolites (measured as Fluorescent Aromatic Compounds – FACs), 7-ethoxyresorufin O-deethylase (EROD) and glutathione S-transferase activity in liver, gill and intestine from the same animals were previously determined in our laboratory (results published in Costa et al., 2011). Biliary BaP metabolites were significantly correlated with liver *CYP1A* mRNA (r=0.91, p=0.004), gill *ABCC2* mRNA (r=0.93, p=0.022), *CYP1A* mRNA (r=0.95, p=0.013) and *GSTα* mRNA (r= 0.90, p=0.036). EROD activity and *CYP1A* mRNA correlations were r=0.95 in liver (p=0.014), r=0.76 in gill (p=0.131) and r=0.86 in proximal intestine (p=0.062).

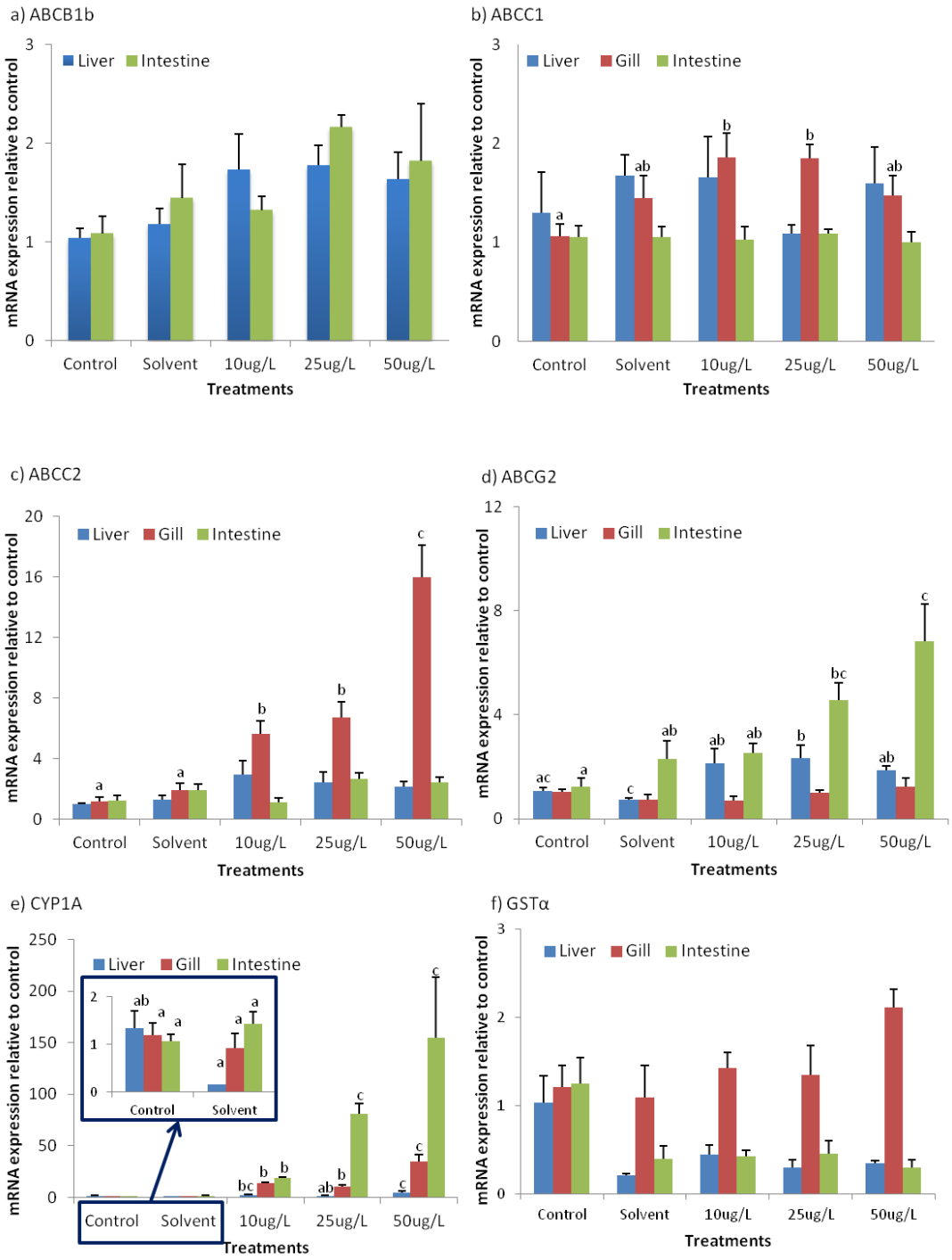

Figure 4.5 – Relative mRNA expression of *ABCB1b* (a), *ABCC1* (b), *ABCC2* (c), *ABCG2* (d), *CYP1A* (e) and *GSTα* (f) in liver, gill and proximal intestine of animals exposed to waterborne BaP.

ABCB1b mRNA expression was not detected in gill. Insert in (e) refers to detailed *CYP1A* mRNA expression in control and solvent. Expression was quantified by qRT-PCR, and fold changes to control were determined using the $2^{-\Delta\Delta CT}$ method. Results are given as mean±SE (n=4). Different letters denote significant differences ($p<0.05$) between treatments within the same tissue.

4.4.4. Dietary exposure to BaP

Gene expression levels of *ABCB1b*, *ABCC1*, *ABCC2*, *ABCG2*, *CYP1A* and *GSTα* of animals from dietary exposure to 100 and 200µg of BaP/g of food are displayed in figure 4.6 (a-f).

In this exposure route, high variability between animals of the same treatment was seen in mRNA expression levels of the ABC transporters. Unexpectedly, expression of *ABCC1* (figure 4.6b) and *ABCG2* (figure 4.6d) in proximal intestine of solvent exposed animals was higher than the levels seen in the remaining treatments. Gill of animals exposed to 100 µg of BaP/g of food had the highest levels of *ABCC2* mRNA expression (figure 4.6c). Moreover, although without significant differences to control groups, *ABCB1b* gene expression in liver increased in animals exposed to 100 and 200 µg of BaP/g of food (figure 4.6a). No significant changes were seen in *GSTα* mRNA expression after Bap exposure (figure 4.6f).

In this assay, the most significant result was the induction of intestinal *CYP1A* mRNA in dietary exposed animals (figure 4.6e), seen after exposure to 100 and 200 µg of BaP/g of food (maximum of 13-fold increase in the latter BaP concentration). Biliary BaP metabolites (measured as FACs), EROD and GST activities in liver, gill and intestine from the same animals were previously determined in our laboratory (results published in Costa et al., 2011). Intestinal *CYP1A* mRNA expression was highly correlated with EROD activity ($r=0.95$, $p=0.048$) and biliary BaP metabolites ($r=0.97$, $p=0.025$).

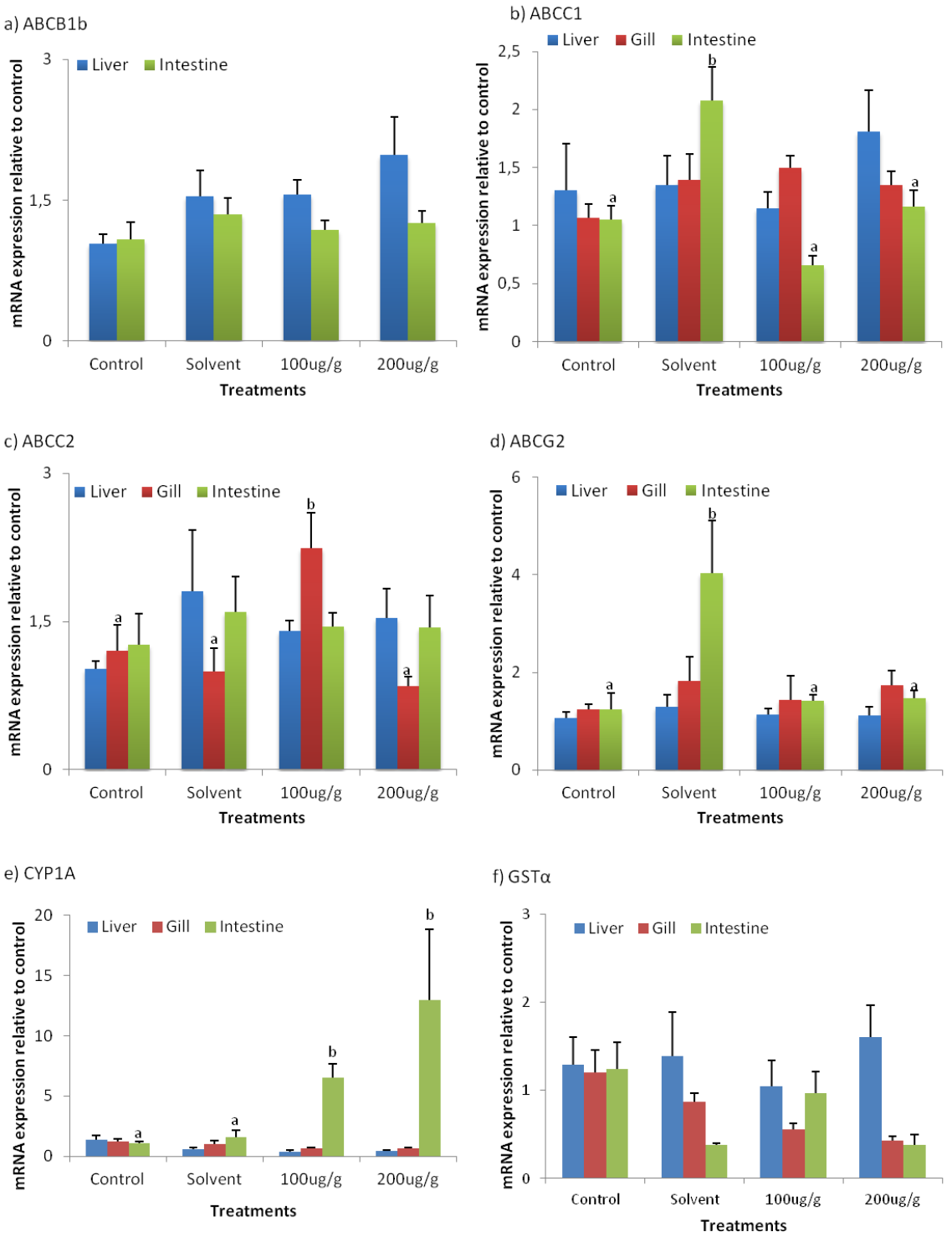

Figure 4.6 - Relative mRNA expression of ABCB1b (a), *ABCC1* (b), *ABCC2* (c), *ABCG2* (d), *CYP1A* (e) and *GSTα* (f) in liver, gill and proximal intestine of animals exposed to dietary BaP.

ABCB1b mRNA expression was not detected in gill. Expression was quantified by qRT-PCR, and fold changes to control were determined using the $2^{-\Delta\Delta CT}$ method. Results are given as mean±SE (n=4). Different letters denote significant differences ($p<0.05$) between treatments within the same tissue.

4.5. Discussion

In this study, expressional changes of ABC transporters (*ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2* and *ABCG2*), *CYP1A* and *GSTα* were determined upon waterborne and dietary BaP exposures in O. niloticus. After the identification of the target partial sequences in this specie, relative mRNA expression was measured in tissues involved with the captation and excretion of pollutants and/or its metabolites (liver, gill and proximal intestine).

4.5.1. Identification and tissue distribution of ABC transporters, *CYP1A* and *GSTα*

Molecular data on Nile tilapia obtained in this study, demonstrated that, with exception of *ABCB1b*, all the remain deduced aa sequences of the target ABC transporters (*ABCB11*, *ABCC1*, *ABCC2* and *ABCG2*), have at least one of the classical features of this protein family, C-Motif (LSGGQ), Walker A (GxxGxGKS/T, where x equals any aa) and/or Walker B (xxxxD, where x equals hydrophobic residues), cytoplasmatic highly conserved amino acid sequences that can be found in all vertebrate ABC proteins (Hyde et al., 1990). Topology analysis (figure 4.2) revealed that partial aa sequences for the ABC transporters identified in this study are analogous to what has been described for other species (Lage 2003; Leslie et al., 2005). Moreover, the high degree of homologies with other fish and mammals seen after multiple alignments (figure 4.1), and clustering of the sequences with other fish species in phylogenetic analysis (figure 4.3) consequently demonstrate the presence of the genes for the ABC transporters in Nile tilapia. In the case of *ABCB1*, two copies of *ABCB1* gene can be found in zebrafish genome, called *ABCB1a* and *ABCB1b* (Annilo et al., 2006). Recently, a novel *ABCB1* isoform was identified in trout and named *ABCB1b* (Fisher et al., 2011). Results show that Nile tilapia sequence has higher degree of identity with zebrafish *ABCB1b* (*ABCB4*), and trout *ABCB1a*, than to zebrafish *ABCB1a* (figure 4.3). Thus, based on the similarities with the isoforms originally named in zebrafish, Nile tilapia *ABCB1* identified in this study should be of type b. Regarding *ABCG2* gene, four isoforms (from a to d) are described in the zebrafish genome (Annilo et al., 2006). Our results have shown that *ABCG2* identified in Nile tilapia has higher degree of homology with zebrafish *ABCG2a* (figure 4.3).

In this study, relative mRNA expression of the different ABC transporters, *CYP1A* and *GSTα* in liver, gill and proximal intestine of *O. niloticus* was assessed. Only a few

studies have described the distribution pattern of ABC transporters in aquatic organisms, namely in different tissues of trout (Zaja et al., 2008a; Zaja et al., 2008c; Loncar et al., 2010), zebrafish (Long et al., 2011a), little skate (Cai et al., 2003), shipshead meadow (Hemmer et al., 1998) and *Poecilia reticulata* (Hemmer et al., 1995). The distribution pattern of *ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2* and *ABCG2* found in Nile tilapia follows the ones described in those studies, and is also in agreement with tissue distribution pattern determined in mammals (Flens et al., 1996; Keppler and Konig, 1997; Gerloff et al., 1998; Mottino et al., 2000; Malliepaard et al., 2001; Brady et al., 2002), with highest expression levels of ABC transporters found in important physiological/pharmacological barriers (Szakács et al., 2008).

ABCB1b and *ABCB11* mRNA expression was not detected in gill, and these results are in agreement to what is described in the literature for other fish species (Zaja et al., 2008a; Loncar et al., 2010). Zaja et al. (2008a) suggested that the high blood content in the gills could potentially mask the gene expression in specific cells types, such as gill epithelial cells, and indicated primary gill epithelial cells as a more adequate model for studying ABC transporters in gills. In subsequent works, *ABCB1* and *ABCB11* were detected at low levels in a gill cell line in trout (Fisher et al., 2011). *ABCB11* mRNA tissue distribution pattern observed in this study, with almost exclusive expression in liver (very low expression was seen in intestine and no expression in gill), is in agreement with its described function of transporter of bile salts from hepatocytes into the bile (Gerloff et al., 1998). Similarly to what has been described for mammals and other fish species, this study has shown that, also in Nile tilapia ABC transporters have distinct mRNA expression patterns in different tissues. In mammals, these different patterns of expression have been shown to be function related (Szakács et al., 2008). Therefore, this might also be true for Nile tilapia, although more research on this matter remains necessary, namely at a post transcriptional level and on cellular localization of ABC transporters in fish. However, this kind of approach is still difficult to achieve since, with exception of ABCB1, no functional antibodies are available for ABC transporters in fish species.

Higher levels of *CYP1A* mRNA expression were seen in liver and gill when comparing to the proximal intestine, with gill showing slightly higher levels of *CYP1A* mRNA expression than liver (figure 4.4). This data is in agreement with previous results reported in *Salmo salar*, where gill tissue had highest basal levels of *CYP1A* mRNA, than liver, kidney and brain (Rees et al., 2003). Considering that mRNA *CYP1A* expression found in tilapia tissues is well correlated with the levels of CYP1A activity measured in the same animals in a previous study (Costa et al., 2011), the higher basal levels of steady state *CYP1A* mRNA found in gill tissue may reflect the biotransformation function of gills,

when the exposure to contaminants is mainly through water (Levine and Oris, 1999; Rees et al. 2003). Additionally, major CYP1A expression and/or activity typically found in liver, is consistent with the role of this organ in xenobiotic metabolism and excretion (Levine and Oris, 1999; Sarasquete and Segner, 2000), whose overall contribution to detoxification is well accepted. *GSTα* mRNA expression was considerably higher in gill, than in liver and intestine (figure 4.4). GST sequences have been isolated and characterized in several fish species, such as plaice (Leaver et al, 1993; Martinez-Lara et al., 2002), largemouth bass (Doi et al., 2004), red-seabream (Konishi et al., 2005), mangrove killifish (Lee et al., 2005), river pufferfish (Kim et al., 2010), common carp (Fu and Xie 2006) and bighead carp (Li et al., 2010). The majority of these works reported high hepatic expression of several GST isoforms, indicating that different GST isoforms are associated with hepatic detoxification of xenobiotics (Kim et al, 2010). Moreover, high intestinal levels of GSTs have also been reported in fish (Kim et al., 2010, Li et al., 2010) and mammals (Landi, 2000), indicating a role of this tissue in xenobiotic breakdown. Studies that compared GSTα expression in fish tissues indicated that this specific isoform has higher expression in liver and intestine than gill in bighead carp (Li et al., 2010) and river pufferfish (Kim et al., 2010), oppositely to what was found in Nile tilapia. Higher *GSTα* mRNA levels in gill reinforce the idea that gills also display an important role in xenobiotic metabolism (Jönsson et al., 2006; Nahrgang et al., 2010; Costa et al., 2011).

4.5.2. ABC transporters CYP1A and GSTα transcriptional responses upon BaP exposure

After exposure to BaP, mRNA levels of some of the studied ABC transporters, and of *CYP1A* were up-regulated in Nile tilapia. The most significant results were seen after water exposure, with increased mRNA expression of *ABCC2* (in gill), *ABCG2* (in liver and proximal intestine) and *CYP1A* (in liver, gill and proximal intestine). Although without significant differences, hepatic and intestinal *ABCB1b* and gill *GSTα* mRNA showed a tendency to increase in animals exposed to waterborne BaP. In dietary assays only intestinal *CYP1A* mRNA was significantly up-regulated in BaP exposed animals.

A small number of studies have addressed the modulation of ABC transporters in liver of fish species, and an increase of the hepatic transcripts of *ABCC2* and *ABCG2* in killifish exposed to PAHs (Paetzold et al., 2009) and of hepatic mRNA *ABCC2* in zebrafish and Antartic fish exposed to heavy metals (Zucchi et al., 2010; Long et al., 2011b) has been reported. Our results follow the pattern of expression seen in those studies, since

ABCC2 and *ABCG2* were the transporters with more pronounced changes to BaP exposure.

CYP1A mRNA up-regulation in liver, gill and proximal intestine of water exposed animals, is consistent to what is described in the literature (Van Veld et al., 1997; Ortiz-Delgado et al., 2005; Ortiz-Delgado et al., 2008). Correlations between *CYP1A* mRNA and CYP1A catalytic activity (EROD) measured in a previous study performed in our laboratory using the same animals (Costa et al., 2011), were high for all tissues, although only significant in the liver (r=0.95, p=0.014), providing evidence that an increase in mRNA expression of *CYP1A* reflects an increase in protein functionality. In Costa et al., 2011, we have demonstrated that waterborne BaP exposure results in high rates of metabolism (by CYP1A enzymes) in liver, gill and intestine, and that total BaP metabolites measured in bile are correlated with CYP1A activity (measured as EROD activity), in the different tissues. Probably, BaP metabolites formed in extra-hepatic tissues are reabsorbed into the blood stream and released in the gall bladder (Kleinow et al., 1998; Costa et al., 2011). Moreover, BaP metabolites profile in plasma and bile of Nile tilapia exposed to BaP in water showed that these are mostly phase II conjugates (Rey-Salgueiro et al., 2011). In our study, high correlations were also found between total BaP metabolites (Costa et al., 2011) and 1) *ABCC2* mRNA expression in gill (r= 0.93, p=0.022), and 2) *ABCG2* mRNA expression in intestine (r=0.85, p=0.066). Although we cannot draw any conclusions about the activity of these transporters, since no protein or functionality studies were done, mRNA up-regulation and the correlations observed with biliary BaP metabolites can suggest that, after water exposure, efflux of phase II BaP metabolites most probably occurs via ABCC2 in gill, and ABCG2 in intestine. Previous studies have reported that ABCC2 efflux of xenobiotics occurs through a co-transport mechanism with reduced glutathione (GSH) (reviewed in Leslie et al., 2005), and the significant correlation seen between *GSTα* and *ABCC2* mRNA in gill further supports this hypothesis. Regarding the intestine, it has been hypothesized that ABC transporter proteins may be involved in the luminal directed transport of BaP metabolites in a human colon adenocarcinoma cell line and ABCB1, ABCC1 and ABCC2 transporters were ruled out as possible candidates involved in this efflux (Buensen et al., 2002; Lampen et al., 2004). However, Ebert et al. (2005) has identified ABCG2 as one of the efflux transporters of BaP conjugates formed in Caco-2 cells back into the intestinal lumen, supporting our results of *ABCG2* mRNA expression in Nile tilapia intestine. Moreover, Mao et al. (2005) reported that ABCG2 seems to preferentially transport sulfated conjugates of steroids and xenobiotics over GSH and glucoronide metabolites, which is in agreement with the lack of correlation seen between mRNA expression of this transporter and GSTα mRNA.

Despite the lack of statistical significance, we also observed an increase in *ABCB1b* transcriptional levels in liver and proximal intestine of water exposed animals. Up-regulation of *ABCB1* in response to xenobiotics is not usually large (1-2 -fold) (Smital et al., 2003), and the individual variability can be masking that response in this study. Although BaP itself has been indicated as a non substrate for ABCB1 in mammals (Schuetz et al., 1998, Buesen et al., 2002), others indicate the opposite (Chao Yeh et al., 1992; Fardel et al., 1996) and the subject remains in discussion. Nevertheless, in a recent work by Zaja et al. (2011) no BaP interaction was seen with ABCB1 activity in a hepatoma cell line of Poeciliopsis lucida. In general, mRNA up-regulation of ABC transporters reported in this study could be a response to the presence of BaP and/or its metabolites. However, more work is necessary at post-transcriptional and protein levels, in order to confirm these hypothesis. Induction of ABC transporters has also been associated with a non-specific general stress response in *Mytilus californianus*, rather than to the presence of specific substrates of those transporters (Eufemia and Epel 2000). In our study, morphological and behavioral indices (data not shown) were not altered in the animals exposed to BaP, which gives us an indication that these animals were not under stressful conditions, and that they were responding to the presence of the contaminant or its metabolites.

As previously stated, no significant changes were seen in ABC transporters after dietary exposures to BaP. Intestinal, but not hepatic, up-regulation of *CYP1*A transcriptional levels can indicate that animals were under BaP induced stress, and that the majority of BaP metabolism will occur at intestinal level, limiting the amount of parent compound that reaches the liver to be metabolized. This type of response has been reported at post-transcriptional levels in liver (Van Veld et al. 1987; Reynolds et al. 2003; Costa et al. 2011) and intestine (McElroy et al., 1992; James et al., 1997; Costa et al., 2011) of fish exposed to dietary PAHs. Moreover, high correlations were seen between intestinal *CYP1A* mRNA and intestinal EROD activity and biliary BaP metabolites (Costa et al., 2011) reinforcing the idea that mRNA expression of *CYP1A* reflects protein functionality, similarly to what was observed in water exposure.

Among the ABC transporters identified in this study, *ABCC1* mRNA expression was not significantly altered by the presence of BaP and/or its metabolites, and was not correlated with the remaining parameters evaluated. Despite our results, much of the *in vitro* evidence indicates that ABCC1 has a role in the removal of toxins as glutathione, glucoronide or sulphate conjugated metabolites from cells (reviewed in Leslie te al., 2001). Additionally, *ABCC1* gene induction was seen in zebrafish exposed to heavy metals (Long et al., 2011a) indicating that this transporter can be involved in heavy metal detoxification in this specie.

In dietary exposure to BaP, high variability was observed within each treatment, probably due to the fact that it is not possible to control the exact amount of food that each animal eats. This variability can be masking a possible response of ABC transporters mRNA expression, which is usually not very large, as opposed to *CYP1A* mRNA response (10-100 fold induction) (Epel et al., 2008; Smital et al., 2003). The induction of intestinal *ABCC2* and *ABCG2* mRNA expression in solvent exposed animals is an unexplainable result, as we cannot input the observed up-regulation to the presence of the solvent, since BaP exposed groups did not show the same response pattern, and were simultaneously exposed to same quantity of solvent. Moreover, this response to the solvent was only seen in these genes and, in waterborne exposure, no such effect was seen for any of the assessed genes.

4.6. Conclusion

In conclusion, partial mRNA sequences of *ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2* and *ABCG2* were identified in *O. niloticus*, and the gene expression tissue distribution pattern of these transporters in Nile tilapia follows the ones already described in mammals and in other aquatic species. Furthermore, novel information about ABC transporters transcriptional levels after controlled exposure to BaP, a ubiquitous contaminant in aquatic environments, in liver, gill and proximal intestine is provided. Up-regulation of *ABCC2* and *ABCG2* transcriptional levels could be related to a possible role of these transporters in the efflux of BaP metabolites in these tissues. New information about ABC transporters provided in this work can be applied in future toxicological or physiological studies, helping to achieve a wider knowledge of the contribution of these proteins in the overall process of xenobiotic detoxification in aquatic species.

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Chapter 5

Tissue distribution and response patterns of Pgp and CYP1A proteins in Nile tilapia (*Oreochromis niloticus*) after waterborne exposure to Benzo(a)pyrene (BaP)

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5. Tissue distribution and response patterns of Pgp and CYP1A proteins in Nile tilapia (*Oreochromis niloticus*) after waterborne exposure to Benzo(a)pyrene (BaP)

5.1. Abstract

The possible correlation between P-glycoprotein (Pgp) and Cytochrome P450 (CYP1A) in the pathway of detoxification of xenobiotic compounds in aquatic organisms is still not well characterized and was under investigation in this study. After waterborne exposure of Nile tilapia (*Oreochromis niloticus*) to different Benzo(a)pyrene (BaP) concentrations, the protein levels and the tissue distribution patterns of Pgp and CYP1A were evaluated by immunochemical approaches. Pgp protein levels and distribution pattern were evaluated with two different mammalian Pgp antibodies (C219 and C494), with different results for each antibody. Similarly to what has been described for other fish species, C219 reacted with a ~170kDa protein in liver samples, and was localized to the bile canaliculi. No positive reaction was seen in gills, and in gut epithelial cells only 1.35% of the samples showed a mild positive reaction. After probing with C494, a positive reaction was seen in liver, gills and intestine, covering a wider set of cell types, with a similar cellular localization described for mammals and other fishes using the same antibody. Levels of Pgp expression were not altered after *in vivo* exposure to BaP. CYP1A was detected with the mAb C10-7 in liver, gills and intestine of Nile tilapia, showing a tissue distribution pattern similar to what has been described for other species and following a BaP dose-dependent fold induction. Taken together, these results suggest that BaP is not itself a substrate for Pgp, while CYP1A is involved in its metabolism in barrier tissues such as liver, gills and intestine.

Keywords: *Pgp, ABCB1, MXR, Nile tilapia, Immunohistochemistry, Western blot*

5.2. Introduction

The toxicity of many xenobiotics is influenced by the target organism's ability to deal with the compounds through detoxification mechanisms, inherently expressed in certain cell types. The multidrug resistance (MDR) membrane P-glycoprotein (Pgp), first discovered in mammalian tumour cells (Juliano and Ling, 1976), is an energy dependent pump that translocates a wide variety of structurally and functionally diverse substrates across cellular membranes (Leslie et al., 2005). This efflux transporter belongs to the highly conserved ATP-Binding Cassette (ABC) superfamily of transmembrane proteins that comprises members with highly diverse physiological functions (Dean and Annilo, 2005). Since some substrates are endo- and xenobiotics, it is believed that Pgp displays an ecotoxicological role in cellular detoxification (Leslie et al., 2005). Research on ABC efflux transporters in aquatic organisms is still limited, although an increasing number of studies conducted to elucidate the presence and function of these proteins in fish species have been made (Bard et al., 2002a; Bard et al., 2002b; Costa et al., 2012; Fischer et al., 2011; Zaja et al., 2008). Another important mechanism of cellular detoxification is the biotransformation of xenobiotics into more easily excreted compounds by enzymatic systems, such as the cytochrome P450 enzymes (CYP). These enzymes, in phase I of biotransformation, convert the chemicals into more hydrophilic forms, through oxidation, reduction or hydrolysis reactions, favouring their excretion (Okey et al., 1986). Cytochrome P4501A (CYP1A) are among the major oxidative enzymes induced in fish and other vertebrates by several types of pollutants, including polycyclic aromatic hydrocarbons (PAHs). CYP1A gene expression, protein levels and activity usually respond in a dose-dependent manner to environmental contamination levels (Buhler and Williams, 1989; Costa et al., 2011; Costa et al., 2012; van der Oost et al., 2003; Van Veld et al., 1997). In light of their functions, some studies have investigated the possibility that Pgp (ABCB1) and CYP1A proteins could act in a coordinated fashion providing an important mean of protection of the cells against xenobiotic insults, due to the existence of common substrates (Bard et al., 2002b; Fardel et al., 1996; Xu et al., 2005). Benzo(a)pyrene (BaP) is a common PAH, widely distributed in aquatic environments, whose carcinogenic and mutagenic properties have been intensively studied (Buhler and Williams, 1989; Tsukatani et al., 2003). Additionally, this xenobiotic is also a known CYP1A inducer in several experimental models (Costa et al., 2011; Costa et al., 2012; Jönsson et al., 2006; Ortiz-Delgado et al., 2005; van der Oost et al., 2003; Van Veld et al., 1997), and has been proposed as a possible substrate for Pgp in mammalian *in vitro* studies (Chao Yeh et al., 1992; Fardel et al., 1996). However, this subject remains a matter of debate, as other

studies report opposing results, both in mammals as in fish (Buesen et al., 2002; Colombo et al., 2003; Schuetz et al., 1998; Zaja et al., 2011). A previous study conducted in our laboratory showed that Pgp mRNA (*ABCB1b*) was not significantly up-regulated in Nile tilapia exposed to waterborne and dietary BaP, although an increasing trend of hepatic and intestinal *ABCB1b* mRNA expression was observed after 14 days of water exposure to this compound (Costa et al., 2012). Tissue distribution patterns of CYP1A and Pgp have been characterized in different experimental models, and the results showed high expression of these proteins in tissues that function as important physiological/pharmacological barriers for the uptake, distribution and elimination of xenobiotics (Hyyti et al., 2001; Sarasquete and Segner, 2000; Szakács et al., 2008). In fishes, as in other vertebrates, liver and intestine are important organs for xenobiotic detoxification, and expression of CYP1A and Pgp has been described for different species (Bard et al., 2002a; Cooper et al., 1999; Costa et al., 2011; Costa et al., 2012; Van Veld et al., 1997). Fish gills also constitute an important physiological barrier in fishes, since they are endowed with biotransformation enzymes, important for the first-pass metabolism of waterborne pollutants, including CYP1A (Costa et al., 2011; Jönsson et al., 2006; Nahrgang et al., 2010). However, the study of the presence and functionality of Pgp in gills of aquatic organisms has giving origin to contradictory results, since some studies in mussels and fish report its presence (Hemmer et al., 1995; Keppler and Ringwood, 2001b; Smital et al., 2003), while Pgp mRNA was not found to be expressed in gills of Nile tilapia (Costa et al., 2012) nor in trout (Loncar et al., 2010; Zaja et al., 2008). Nile tilapia, *Oreochromis niloticus*, is one of the most important and commonly used species of cichlid fish, not only for fresh water aquaculture (FAO 2004), but also for research in a wide range of scientific areas (Almeida et al., 2001; Costa et al., 2011; Costa et al., 2012; Lee et al., 2005; Rey-Salgueiro et al., 2011) and was used as the model organism in this study. This study was conducted to further elucidate the tissue distribution patterns and the possible correlations of Pgp and CYP1A in Nile tilapia, after waterborne exposure to BaP. To fulfill our objectives we have used an immunohistochemical approach and the quantification of the protein level, by western blot technique, in tissues involved with the uptake and detoxification of xenobiotics (liver, gills and proximal intestine), after waterborne exposure to BaP.

5.3. Materials and methods

5.3.1. Animals

Oreochromis niloticus used in this study were born and raised in the laboratory (CIIMAR, Porto, Portugal). Animals used were juveniles, and therefore not sexually mature, as confirmed by macroscopic analysis of the gonads at sampling. Until the start of the exposure experiment, fish were kept in 60 L aquaria supplied with biological filtration. Prior to the experiment, animals were randomly distributed in the experimental aquaria (30 L), and submitted to an acclimatation period of one week. All tanks were supplied with continuous aeration to maintain dissolved oxygen near saturation. Dechlorinated tap water was used at a temperature of 20±2 ºC, with a 12h:12h (light:dark) photoperiod. Fish were fed commercial food pellets (Aquasoja, Portugal), until satiation, once a day.

5.3.2. Water exposure to BaP

Stock solutions of BaP were prepared in acetone (0.5, 1.25 and 2.5 g/L) and were administered directly to the experimental aquaria. The percentage of solvent added to the experimental aquaria was 0.002%. For the water exposure, juvenile Nile tilapia (N=20, average mass of 16.36±0.82 g, average length of 9.99±0.19 cm) were exposed to nominal water concentrations of 10, 25 and 50 µg of BaP/L for 14 days. Also a solvent control group (acetone alone) was maintained. The tested concentrations have been found in pore waters of estuaries polluted with petrochemical products (Maskaoui et al. 2002). Waterborne exposures were conducted in semi-static conditions in 30 L aquaria. Daily, 80% of the water was renewed, followed by the addition of fresh BaP solution or solvent to each one of the treatment groups. Animals were fed to satiation every other day, and were not fed the day before sampling.

5.3.3. Sampling

Sampling was performed at days 7 and 14 after the beginning of the exposure to BaP. Fish were anesthetized on ice cold water and sacrificed by decapitation. Liver, gills and proximal intestine (first one third of the intestine) were excised, and portions of each tissue were stored at -80ºC (for western blot analysis) or fixed in 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) for 24 hours at 4ºC (for immunofluorescence analysis). Immunofluorescence samples were washed in 50% ethanol (3 X 20 minutes) followed by 70 % ethanol (3 X 20 minutes) prior to storage in 70% ethanol until further use.

5.3.4. Membrane vesicle and microsomes preparation

Plasma membrane vesicles and microsomes were prepared according to the method described by Zaja et al. (2008) with minor modifications. Briefly 50-70 mg of tissue were sonicated (40A for 20 seconds) in 1ml of vesicle buffer (10mM Tris, 250mM sucrose, 0.2mM CaCl2, 1mM Na_2EDTA, 0.1mM PMSF, pH 7.5), centrifuged (1000 x g, 10min, 4ºC) and the supernatant layered on 35% sucrose. Samples were centrifuged at 16000 x g, 30min at 4ºC using SW32 Ti rotor (Beckamn Coulter) and the layer formed at the top of 35% sucrose was collected, diluted in sample buffer (10mM tris, 250mM sucrose, pH 7.5) and centrifuged at 100000 x g (90Ti rotor, Beckman Coulter) for 1 hour at 4ºC. Pellets were ressuspended in 100 µl of sample buffer.

5.3.5. Antibodies

The ABCB1 mouse monoclonal antibody C219 (Gene Tex Inc.) recognizes internal, highly conserved amino-acid sequences, VQEALD and VQAALD, corresponding to the C-terminal and N-terminal regions, respectively found in both human MDR1 and MDR3 isoforms (predicted molecular weight of ~170kDa) (Georges et al., 1990; van den Elsen et al., 1999). It also recognizes the ~170 kDa sister of P-glycoprotein (Spgp, ABCB11) (Childs et al., 1995). The ABCB1 mouse monoclonal antibody C494 (Gene Tex Inc.) is specific for the human MDR1 isoform, and does not cross reacts with MDR3 (Georges et al., 1990). It has been shown to cross-react with Pyruvate Carboxylase, an abundant mitochondrial enzyme (130 kDa), on both immunoblots and immunohistochemical tissue

sections of mammals (Rao et al., 1994). Mouse monoclonal antibody C10-7 (Abcam plc, Cambridge, UK) reacts with hepatic CYP1A protein (~55kDa) from rainbow trout (*Oncorhynchus mykiss*). It has also shown a positive reaction with CYP1A in different tissues of *Spaurus aurata* (Ortiz-Delgado et al., 2005).

5.3.6. SDS-Page and Western blotting

Protein content in all samples was determined according to the method described by Lowry et al. (1951). Samples were diluted in Laemmli buffer 2X (1:1). Non-reduced (no dithiothreitol added in Laemmli buffer) and non-denaturated samples (no boiling prior to the gel loading, no sodium dodecyl sulphate, SDS, in migration buffer) were used for probing with C219, while reduced and denaturated samples were used for probing with CYP1A. Five micrograms (for CYP1A - C10-7) or thirty micrograms (for ABCB1 - C219) of protein were separated by electrophoresis in 6% SDS - polyacrilamide gel (SDS-Page), using a Hoefer SE 250 system (GE Healthcare). Protein bands were transferred to nitrocellulose membranes (Hybond ECL, GE Healthcare), using a wet transfer unit (Hoefer TE 22, GE Helthcare). Membranes were rinsed in TTBS (for C10-7, 0.05% tween 20 in tris-buffered-saline, pH 7.4) or TPBS (for C219, 0.05% tween 20 in phosphate-buffered-saline), and blocked for 1 hour at room temperature in 5% skim milk diluted in the corresponding washing buffer. After rinsing in washing buffer, membranes were probed either with C10-7 (Abcam plc) or C219 (GenTex, Inc) diluted 1/500 in blocking buffer (5% skim milk in TTBS or TPBS), overnight at 4ºC. Membranes were rinsed for 5, 10 and 15 minutes in washing buffer, and incubated with a Horseradish Peroxidase (HRP) conjugated secondary antibody (HRP-Goat anti-mouse IgG, Invitrogen) diluted 1/20000 in washing buffer, for 1 hour at room temperature. After final washes of 5, 10 and 15 minutes in the correspondent washing buffer, signal was detected by enhanced chemiluminescence (Immobilon western chemiluminescent HRP substrate, Millipore). The mammalian mAb C494, directed to Pgp, was tested but no specific reactivity was detected in Nile tilapia tissues applying this antibody by the western blot technique. Images were visualized using a LAS 4000 mini imaging system (Fujifilm) and quantification was performed using Multi gauge software, Fuji Photo Film Co., ltd.

5.3.7. Immunofluorescence microscopy

Tissues stored in 70% ethanol were dehydrated in an ethanol series, cleared in clear-rite 3 (Richard Allen Scientific) and infiltrated and embedded in paraffin (Type 6, Richard Allen Scientific). Paraffin sections (3µm) were collected onto 3-aminopropyltriethoxysilane (Sigma Aldrich) coated slides, air dried and de-waxed in clear-rite 3. Sections were processed for immunofluorescence according to Wilson et al. (2007) with some minor modifications. After antigen retrieval (30 minutes at 98ºC in 0.05% citacronic anhydride) the sections were circled with a hydrophobic pen (PAP pen, Sigma-Aldrich) and blocked with 5% normal goat serum in 1%BSA, 0.05% sodium azide in TPBS, pH 7.4, for 20 minutes at room temperature. Sections were then incubated in a humidity chamber with primary antibodies to CYP1A (C10-7, 1/500) and Pgp (C219, 1/50; C494, 1/50) diluted in 1%BSA/TPBS/0.05% sodium azide, pH 7.4, overnight at 4ºC. Following washes in TPBS (5, 10 and 15 minutes) in a coplin jar, slides were incubated in a humidity chamber with goat anti-mouse Alexa Fluor 568 conjugated secondary antibody (Invitrogen) diluted 1/400 in TPBS, for 1 hour at 37ºC. After a 5 minute wash in TPBS, the nuclei were stained with DAPI (4´6-diamino-2-phenylindole) for 10 minutes and slides were rinsed in TPBS for 15 minutes. Coverslips were mounted using a glycerol-based fluorescence mounting media (10% mowiol, 40% glycerol, 0.1% 1,4-diazabicyclo[2.2.2]octane (DABCO), 0.1M Tris, pH 8.5). In each slide a control section was included with dilution buffer (1%BSA/TPBS/0.05% sodium azide, pH 7.4) instead of the primary antibody. Sections were immediately observed in a Leica DM6000 B wide field epi-fluorescence microscope, and images captured using a cooled digital camera (Leica DFC340FX) along with the corresponding differential interference contrast (DIC) image. Plates were assembled using Adobe Photoshop CS3 software, and images enhanced while maintaining the integrity of the data. A subjective evaluation of staining intensity in sections probed with the different tested antibodies (C219, C494 and C10-7) was performed according to the method described by Van Veld et al. (1997). Staining was assigned a numeric value of 0 (negative), 0.5 (negative/mild), 1 (mild), 1.5 (mild/moderate), 2 (moderate), 2.5 (moderate/high) or 3 (high) according to its intensity. Negative values indicate that no staining was visible. Values obtained for each treatment in each cellular type were average, and data presented as mean±SE.

5.3.8. Statistical analysis

Treatment and time effects were evaluated by means of a two-way ANOVA, followed by a multiple comparison test (Tukeys test) at a 5% significance level. Some data had to be log or square root transformed in order to fit ANOVA assumptions. All tests were performed using the software Statistica 7 (Statsoft, Inc., 2001).

5.3.9. Ethics statement

The animals used in the research that is described in this paper were treated in accordance with the Portuguese Animals and Welfare Law (Decreto-Lei nº 197/96) approved by the Portuguese Parliament in 1996 and with the European directive 2010/63/UE approved by the European Parliament in 2010. Institutional animal approval by CIIMAR/UP and Direcção Geral Veterinária was granted for this study.

5.4. Results

5.4.1. Western blot detection of P-glycoprotein

Western blot results showed the presence of a protein in Nile tilapia liver that was cross-reactive with the mammalian mAb C219. A single band of, approximately, 170 kDa was detected in liver membrane vesicles both from solvent control and BaP exposed animals, but no immunoreactivity was seen in gill nor in proximal intestine. No significant differences were seen in the reaction intensity between solvent control and BaP exposed animals (figure 5.1).

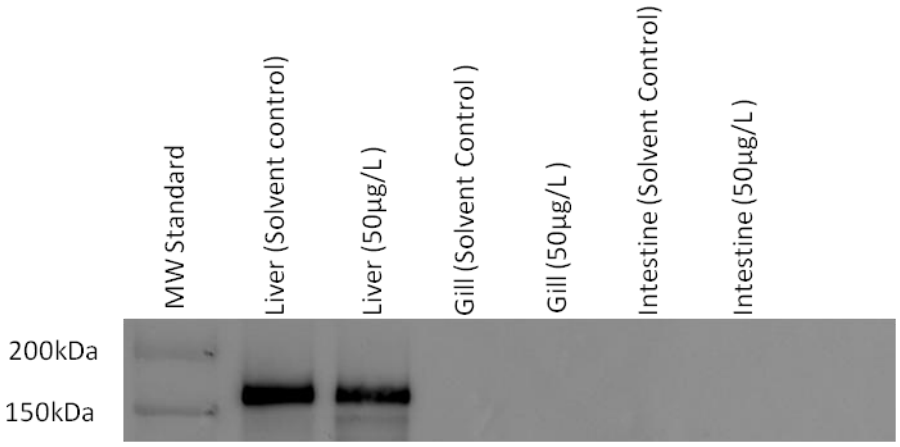

Figure 5.1 - Western blot of membrane vesicles from liver, gill and proximal intestine of Nile tilapia.

Samples from solvent control animals and animals exposed for 14 days to 50µg/L of waterborne BaP. Blots were probed with the mammalian mAb C219 for P-glycoprotein. Five µl of molecular weight (MW) standard were used. Thirty µg of protein were loaded in each lane.

5.4.2. Cellular localization of P-glycoprotein

The cellular localization of Pgp was investigated by immunofluorescence in liver, gill and proximal intestine of Nile tilapia exposed to different concentrations of waterborne BaP for 7 and 14 days. The mammalian mAbs C494 and C219 were used to probe the tissue sections, and different results were seen with each antibody (table 5.1, figure 5.2).

Liver

After labelling with C494, a positive reaction was seen in plasma membrane, hepatocytes cytoplasm, vascular endothelium and bile canaliculi from liver samples (table 5.1, figure 5.2a). Frequency of staining in bile canaliculi and vascular endothelium was very consistent and all liver samples showed a positive labelling. Staining of the plasma membrane showed a high frequency between samples analysed, although there was some variety in the number of hepatocytes that showed this particular labelling. Some tissue sections showed a higher percentage of labelled hepatocytes than others, without any clear correlation pattern with the exposure to BaP. Cytoplasm of the hepatocytes presented a weaker but consistent labelling (figure5.2a). The C219 labelling was confined

to biliary canaliculi, with consistent frequency of staining (figure 5.2b). No other cell types showed a positive reaction with C219. For both antibodies, no significant differences to control were seen in the staining intensity in tissues of animals exposed to the different BaP concentrations after 7 and 14 days of exposure (table 5.1). No positive reaction was seen in control sections (dilution buffer only).

Gill

In gill sections labelled with C494, positive staining was observed in several cell types (pillar cells, epithelial cells, chloride cells and undifferentiated cells) as described in table 5.1 and figure 5.2c. Frequency of staining was consistent throughout the samples analysed, and staining intensity varied from mild/moderate to high in the different cell types (table 5.1). No significant differences were observed between control and samples from animals exposed to the different concentrations of BaP at 7 and 14 days of exposure. No positive labelling was observed in gill sections probed with C219 (figure 5.2d). In some tissue sections, including control (dilution buffer only), some samples showed a mild cross-reactivity of erythrocytes. No other cell types showed a positive reaction in control sections.

Proximal intestine

Probing with C494 resulted in a positive labelling in enterocytes and vascular endothelium of proximal intestine, as described in table 5.1 and figure 5.2e. Frequency of staining was consistent, since we observed a positive reaction in all samples analysed. In general, labelling intensity increased from the base of the crypts to the tips of the villi (figure 5.2e). No significant differences to control were seen in staining intensity in proximal intestine of animals exposed to the different concentrations of BaP during the 7 and 14 days of exposure (table 5.1). In 1.35% of the samples analysed, a mild reaction was detected in the apical membrane of enterocytes after probing with C219 (figure 5.2f) while for all the remaining samples no positive reaction was observed (table 5.1). Samples that showed positive reaction with C219 were randomly distributed throughout the different treatments (solvent control, 10 and 50 µg/L of BaP), indicating that there was no correlation of the labelling with the exposure to BaP.

Table 5.1 - Cellular localization of Pgp in liver, gill and proximal intestine of Nile tilápia exposed to different concentrations of waterborne BaP.

Samples were probed with two different Pgp mammalian antibodies, C219 and C494. Values are reported as mean immunofluorescence staining score ± SD

Tissue	Cell type	C219								C494							
		Day 7				Day 14				Day 7				Day 14			
		Solvent Control	10µg/L	25µg/L	50 g/L	Solvent Control	10µg/L	25µg/L	50µg/L	Solvent Control	10µg/L	25µg/L	50µg/L	Solvent Control	10µg/L	25µg/L	50µg/L
Liver	Hepatocytes cytoplasm	0	0	0	0	0	0	0	0	1.7±0.3	1.8±0.3	2.2±0.3	1.3±0.9	1.2±0.3	1.8±0.3	1.3±0.3	1.6±0.3
	Plasma membrane	0	0	0	0	0	0	0	0	2.0±0.0	2.1±0.3	2.0±0.9	1.6±1.1	1.0±1.1	1.4±1.4	1.2±1.0	1.4±0.9
	Bile canaliculi	2.0±1.0	2.7±0.3	2.2±0.3	2.5±0.5	2.2±0.8	2.3±0.6	2.3±0.3	2.8±0.4	2.5±0.0	2.5±0.0	2.5±0.0	2.0±0.7	1.3±1.1	2.6±0.5	2.0±0.9	1.3±1.2
	Vascular endothelium	0	0	0	0	0	0	0	0	2.3±0.3	2.5±0.6	1.8±0.8	2.1±0.9	1.7±1.4	2.6±0.5	2.5±0.9	1.3±1.2
Gill	Pillar cells	0	0	0	0	0	0	0	0	2.3±0.3	2.3±0.6	2.7±0.6	2.7±0.3	2.0±0.6	2.3±0.5	2.5±0.5	2.0±0.5
	Epithelial cells	0	0	0	0	0	0	0	0	2.3±0.3	2.3±0.6	2.3±0.6	2.7±0.3	2.1±0.5	2.0±0.7	2.5±0.5	1.8±0.3
	Chloride cells	0	0	0	0	0	0	0	0	2.8±0.3	2.8±0.3	2.7±0.6	2.7±0.3	2.6±0.3	2.3±0.9	3.0±0.0	1.8±0.3
	Undiferentiated cells	0	0	0	0	0	0	0	0	1.7±0.3	2.3±0.6	2.3±0.6	2.7±0.3	1.8±0.3	1.9±0.6	2.5±0.5	1.8±0.3
Proximal intestine	Apical membrane	0.2±0.3	0.3±0.4	0	0.5±0.9	0.2±0.3	0.4±0.8	0	0	2.7±0.3	2.8±0.4	2.8±0.3	2.8±0.3	2.3±0.3	2.6±0.5	2.8±0.3	2.5±0.0
	Enterocytes	0	0	0	0	0	0	0	0	2.3±0.6	2.3±0.4	1.4±0.9	1.4±0.9	1.5±0.5	1.4±1.1	1.9±0.3	2.0±0.0
	Submucosal regions	0	0	0	0	0	0	0	0	2.2±0.8	2.3±0.4	2.3±0.5	2.3±0.5	1.0±0.5	0.9±0.8	1.6±0.6	2.2±0.3

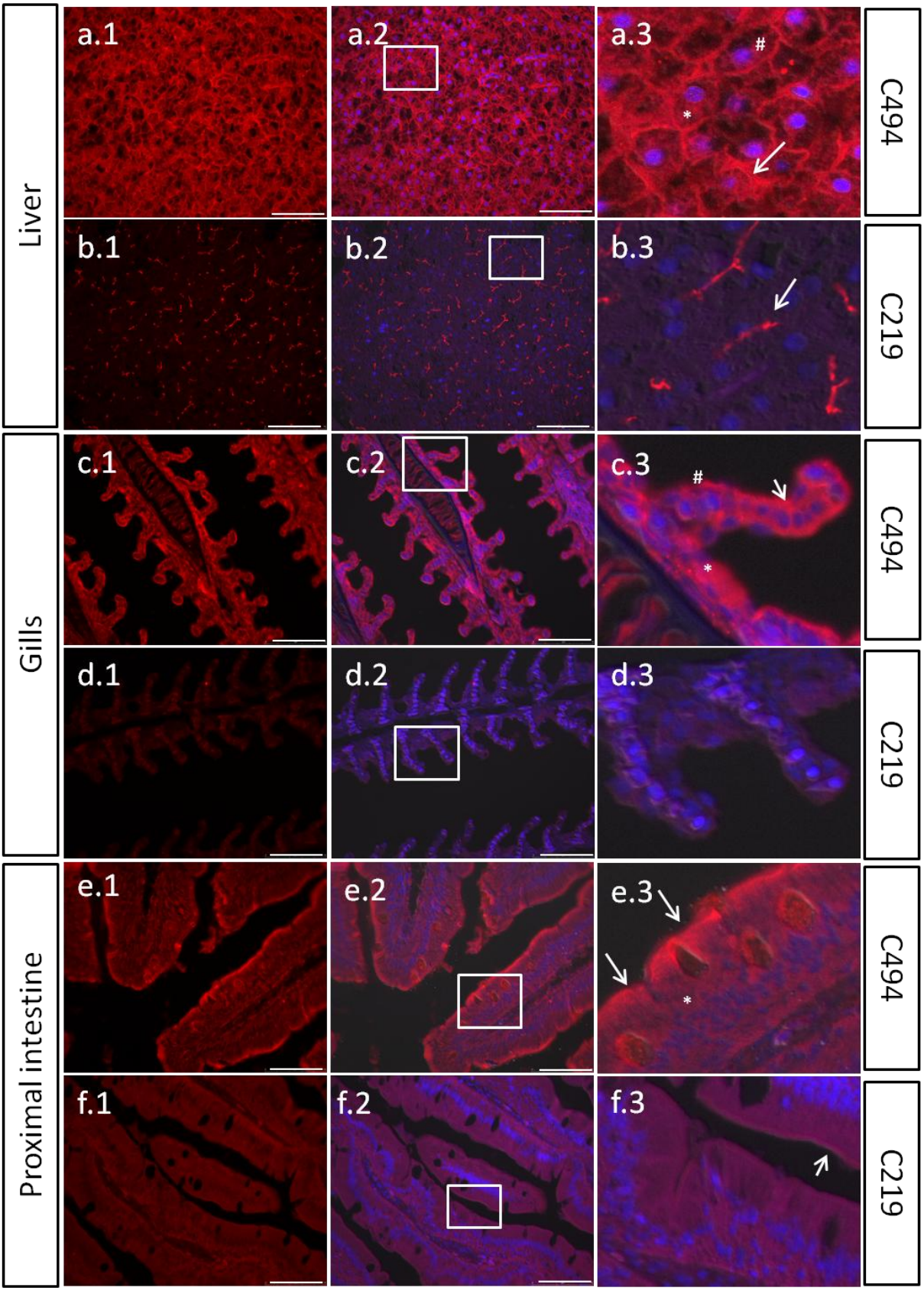
Liver
Gills
Proximal intestine
a.1
a.2
a.3
b.1
b.2
b.3
c.1
c.2
c.3
d.1
d.2
d.3
e.1
e.2
e.3
f.1
f.2
f.3
C494
C219
C494
C219
C494
C219

Figure 5.2 - Representative immunofluorescence results of liver, gill and proximal intestine in Nile tilapia probed with mammalian Pgp antibodies, C219 and C494.

Liver sections stained with C494 (a) and C219 (b), showing positive staining in bile canaliculi (white arrows), hepatocytes cytoplasm (*) and hepatocytes plasma membrane (#). Gill sections stained with C494 (c) and C219 (d), showing positive labelling for C494 in pillar cells (white arrows), epithelial cells (#) and undiferenciated cells (*). Proximal intestine sections stained with C494 (e) and C219 (f), showing positive labelling in plasma membrane (white arrows) and enterocytes (*). To provide structural information, blue nuclei were counterstained with DAPI and DIC image was overlaid (2), image (3) represents a higher magnification of square showed in (2). Scale bar = 75µm.

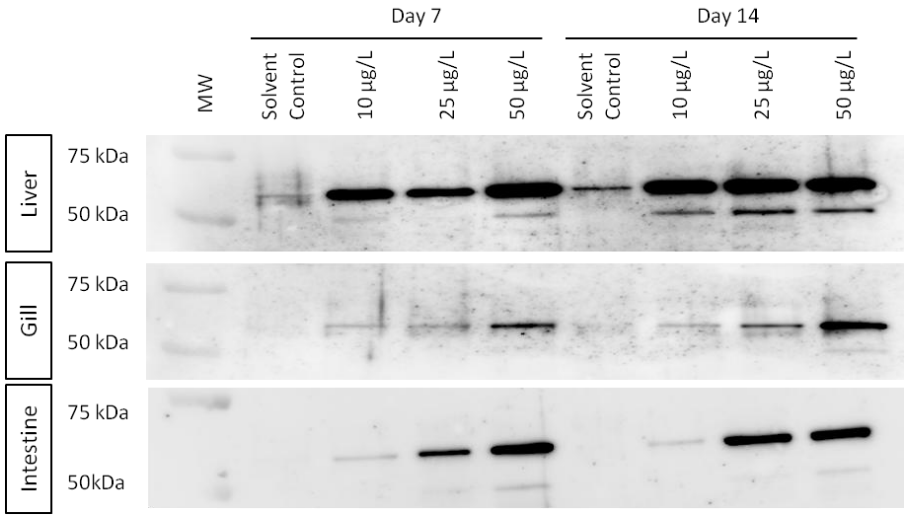

Figure 5.3 - Representative immunoblots of Nile tilapia CYP1A protein in liver, gills and proximal intestine after waterborne exposures to BaP (10µg/L, 25µg/L and 50µg/L) for 7 and 14 days.

Five µg of protein were loaded in each lane and probed with C10-7 mAb. Five µl of molecular weight marker (MW) were loaded in first lane.

5.4.3. Western blot detection of CYP1A

The cross-reactivity of the monoclonal antibody C10-7 with CYP1A protein in Nile tilapia tissues was verified by western blot, using microsomal fractions of liver, gill and proximal intestine. The antibody detected one strong band of approximately 55 kDa, and a secondary weaker band of about 50kDa in heavily reactive samples (figure 5.3).

In general, CYP1A reactivity increased with the concentration of BaP in the three tissues analysed (figures 5.3 and 5.4). In liver, CYP1A protein levels were significantly higher than solvent control in all BaP treatments (both at 7 and 14 days of exposure) ($p<0.05$) (figure 5.4a). Both in gill as in proximal intestine, differences of CYP1A protein levels in BaP exposed animals (to all BaP concentrations, both at 7 and 14 days of exposure) in comparision to control were not statistically significant (figure 5.4b and 5.4c). Maximum fold inductions of CYP1A protein levels were observed in intestine (175.6±75.2; 7 days of exposure to 50 µg/L of BaP), followed by gill (16.2±11.2; 14 days of exposure to 50 µg/L of BaP) and liver (15.6±1.2; 14 days of exposure to 50 µg/L of BaP). No significant differences in CYP1A reactivity were observed, within each treatment, between days 7 and 14 for none of the tissues. In unexposed animals (solvent control) levels of CYP1A protein were higher in liver, followed by gill and intestine with respective values of 3778.6±1478.5, 1306.9±963.9 and 98.9±97.5 arbitrary units (mean±SE).

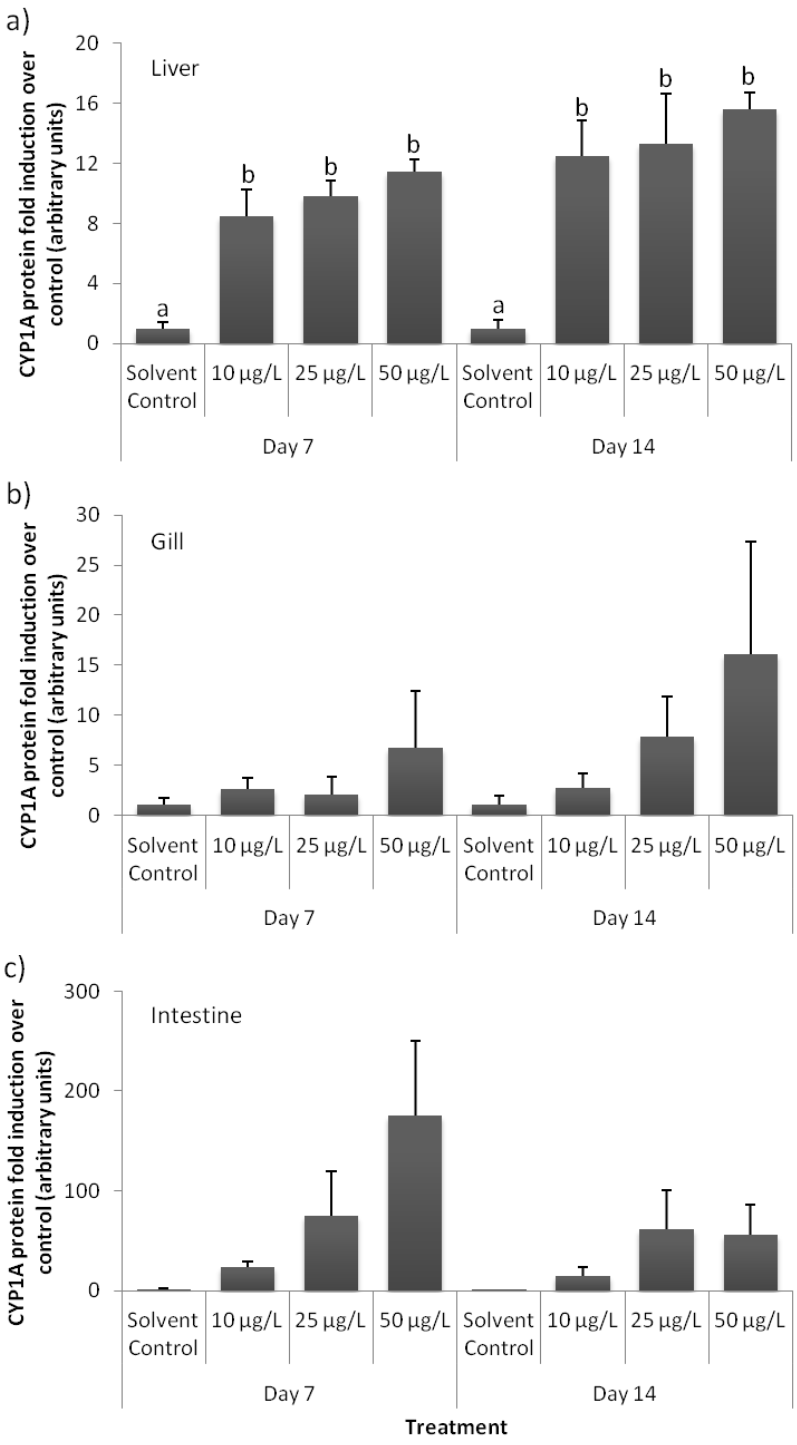

Figure 5.4 - Western blot results of CYP1A protein fold induction over control after Nile tilapia waterborne exposure to BaP in liver (a), gill (b) and proximal intestine (c).

Different letters denote significant differences over the solvent control ($p<0.05$). Results presented as mean±SE. n=3-4.

Table 5.2– Cellular localization of CYP1A in liver, gill and proximal intestine of Nile tilápia exposed to different concentrations of waterborne BaP.
Samples were probed with CYP1A mAb C10-7. Values are reported as mean immunofluorescence staining score ± SD.

Tissue	Cell type	Day 7				Day 14			
		Solvent control	10µg/L	25µg/L	50µg/L	Solvent control	10µg/L	25µg/L	50µg/L
Liver	Hepatocytes cytoplasm	0	1.3±0.3	2.0±0.0	2.2±0.6	0	1.5±0.5	1.5±0.4	2.2±0.8
	Vascular endothelium	0	1.0±0.9	2.0±0.0	1.7±0.6	0	1.0±1.0	1.3±0.9	1.0±1.7
Gill	Pillar cells	0	1.9±0.3	2.3±0.4	2.7±0.3	0	2.4±0.8	3.0±0.0	2.5±0.6
	Epithelial cells	0	0.4±0.8	0.5±0.7	1.3±0.6	0	0.8±0.9	0.7±0.8	1.0±1.2
Proximal intestine	Enterocytes	0	1.3±0.4	1.5±0.6	1.3±1.2	0	1.5±1.0	1.4±0.9	1.8±0.9
	Vascular elements	0	2.3±0.4	2.0±0.4	2.5±0.0	0	2.3±0.3	2.3±0.3	2.8±0.5

5.4.4. Cellular localization of CYP1A

In liver, gills and proximal intestine of solvent control fish no immunoreactivity of CYP1A protein was detected both at days 7 and 14 (table 5.2, figures 5.5, 5.6 and 5.7). Animals´ exposure to BaP resulted in positive labelling of CYP1A protein both in hepatocytes cytoplasm and hepatic vascular endothelium (table 5.2, figure 5.5). Hepatocyte staining was visible in all samples from exposed animals, while a lower frequency of positive labelling was observed in endothelial cells (75% of the samples). Intensity of staining showed an increase with the concentration of BaP, while no differences were seen with the increase of exposure time (from 7 to 14 days) (figure 5.5, table 5.2). Hepatocytes staining intensity varied from negative (solvent control) to moderate/high (50µg/L of BaP), and vascular endothelium staining varied from negative (solvent control) to mild/moderate (50 µg/L of BaP) (table 5.2). In gills, exposure to BaP resulted in CYP1A labelling in pillar cells, with all samples showing a positive reaction (table 5.2, figure 5.6). Epithelial gill cells also stained positively for CYP1A protein after BaP exposure, although showing a lower frequency of staining (55% of the samples). Overall, an increase in the staining intensity was seen with the increase of the concentration of BaP in gill pillar and epithelial cells (table 5.2, figure 5.6), although without statistical significance. Higher staining intensity was seen in pillar cells (from negative to high) than epithelial cells (from negative to mild/moderate) (table 5.2). In intestine, exposure to BaP resulted in positive CYP1A labelling of gut epithelial cells and gut vascular elements (table 5.2, figure 5.7) in all samples analysed. Staining intensity was slightly higher in vascular elements (from moderate to high) than enterocytes (from mild to moderate) (table 5.2). No significant differences were seen in the intensity of staining after exposure to the different concentrations of BaP tested, nor with the time of exposure. In control sections of all tissues (dilution buffer only) no positive immunoreaction was observed.

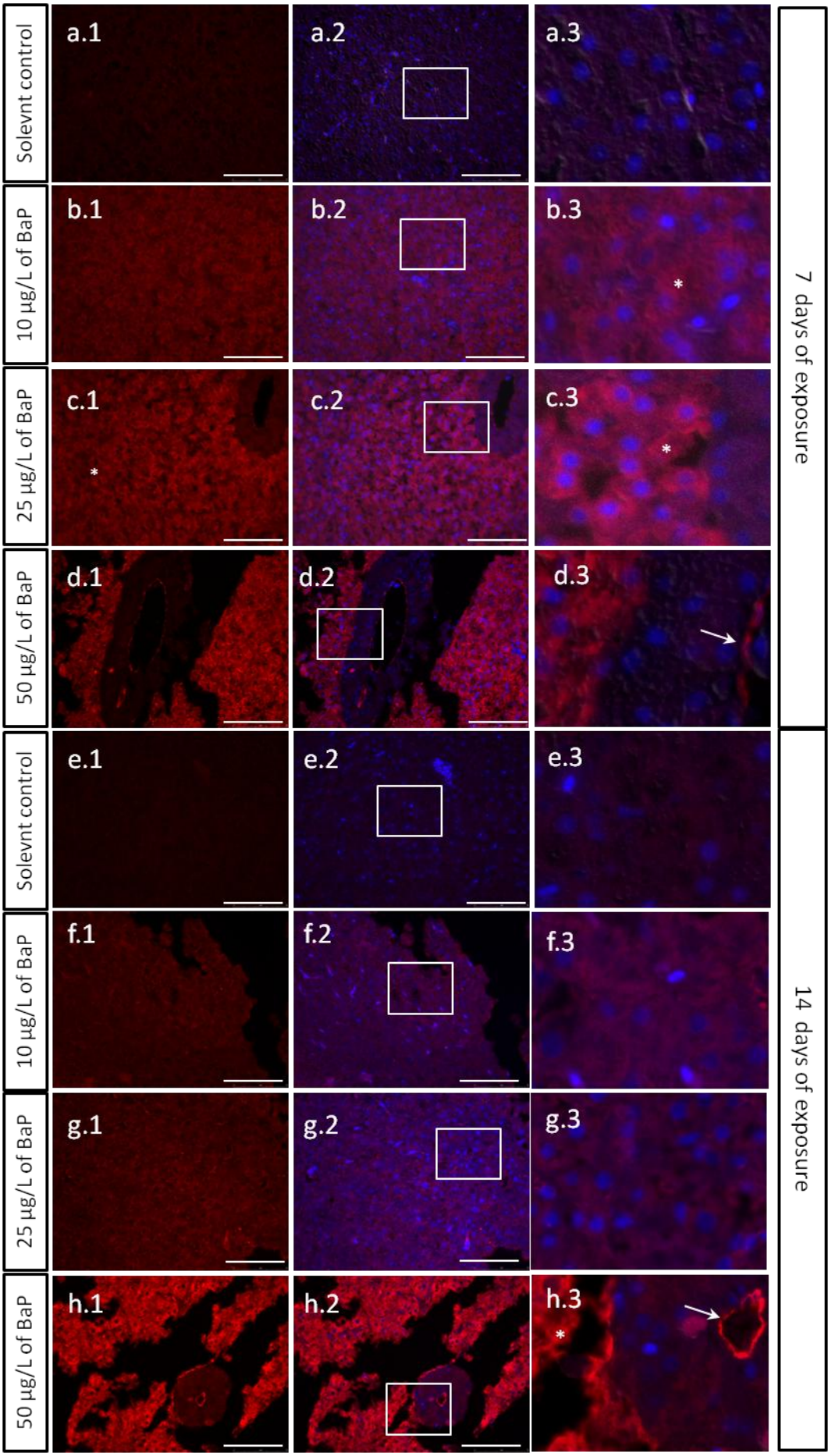
Solevnt control
10 µg/L of BaP
25 µg/L of BaP
50 µg/L of BaP
Solevnt control
10 µg/L of BaP
25 µg/L of BaP
50 µg/L of BaP
a.1
a.2
a.3
b.1
b.2
b.3
c.1
c.2
c.3
d.1
d.2
d.3
e.1
e.2
e.3
f.1
f.2
f.3
g.1
g.2
g.3
h.1
h.2
h.3
7 days of exposure
14 days of exposure

Figure 5.5 - Representative CYP1A immunostaining in liver samples of Nile tilapia exposed to BaP.

(a) 7 days of exposure, solvent control; (b) 7 days of exposure to 10µg/L of BaP; (c) 7 days of exposure to 25µg/L of BaP; (d) 7 days of exposure to 50µg/L of BaP; (e) 14 days of exposure, solvent control; (f) 14 days of exposure to 10µg/L of BaP; (g) 14 days of exposure to 25µg/L of BaP; (h) 14 days of exposure to 50µg/L of BaP. To provide structural information, blue nuclei were counterstained with DAPI and DIC image was overlaid (2), image (3) represents a higher magnification of square showed in (2). White arrows indicate positive labelling in vascular elements, (*) indicate positive labeling in hepatocytes cytoplasm. Scale bar = 75µm.

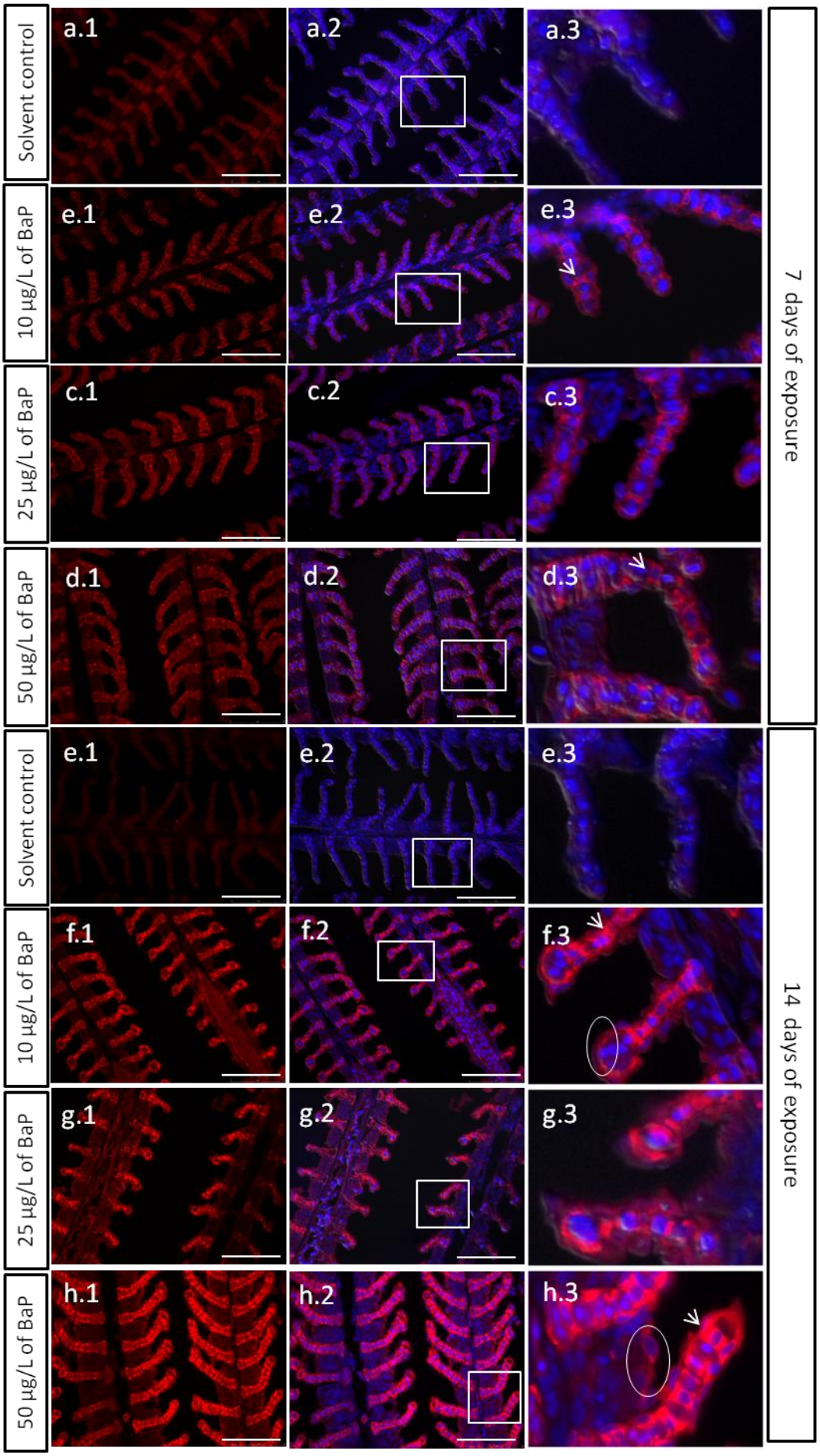
Solvent control
10 µg/L of BaP
25 µg/L of BaP
50 µg/L of BaP
7 days of exposure
Solvent control
10 µg/L of BaP
25 µg/L of BaP
50 µg/L of BaP
14 days of exposure
a.1
a.2
a.3
e.1
e.2
e.3
c.1
c.2
c.3
d.1
d.2
d.3
e.1
e.2
e.3
f.1
f.2
f.3
g.1
g.2
g.3
h.1
h.2
h.3

Figure 5.6 - Representative CYP1A immunostaining in gill samples of Nile tilapia exposed to BaP.

(a) 7 days of exposure, solvent control; (b) 7 days of exposure to 10µg/L of BaP; (c) 7 days of exposure to 25µg/L of BaP; (d) 7 days of exposure to 50µg/L of BaP; (e) 14 days of exposure, solvent control; (f) 14 days of exposure to 10µg/L of BaP; (g) 14 days of exposure to 25µg/L of BaP; (h) 14 days of exposure to 50µg/L of BaP. To provide structural information, blue nuclei were counterstained with DAPI and DIC image was overlaid (2), image (3) represents a higher magnification of square showed in (2). White arrows indicate positive labelling of gill pillar cells. White circles indicate immunolabelling in gill epithelial cells. Scale bar = 75µm.

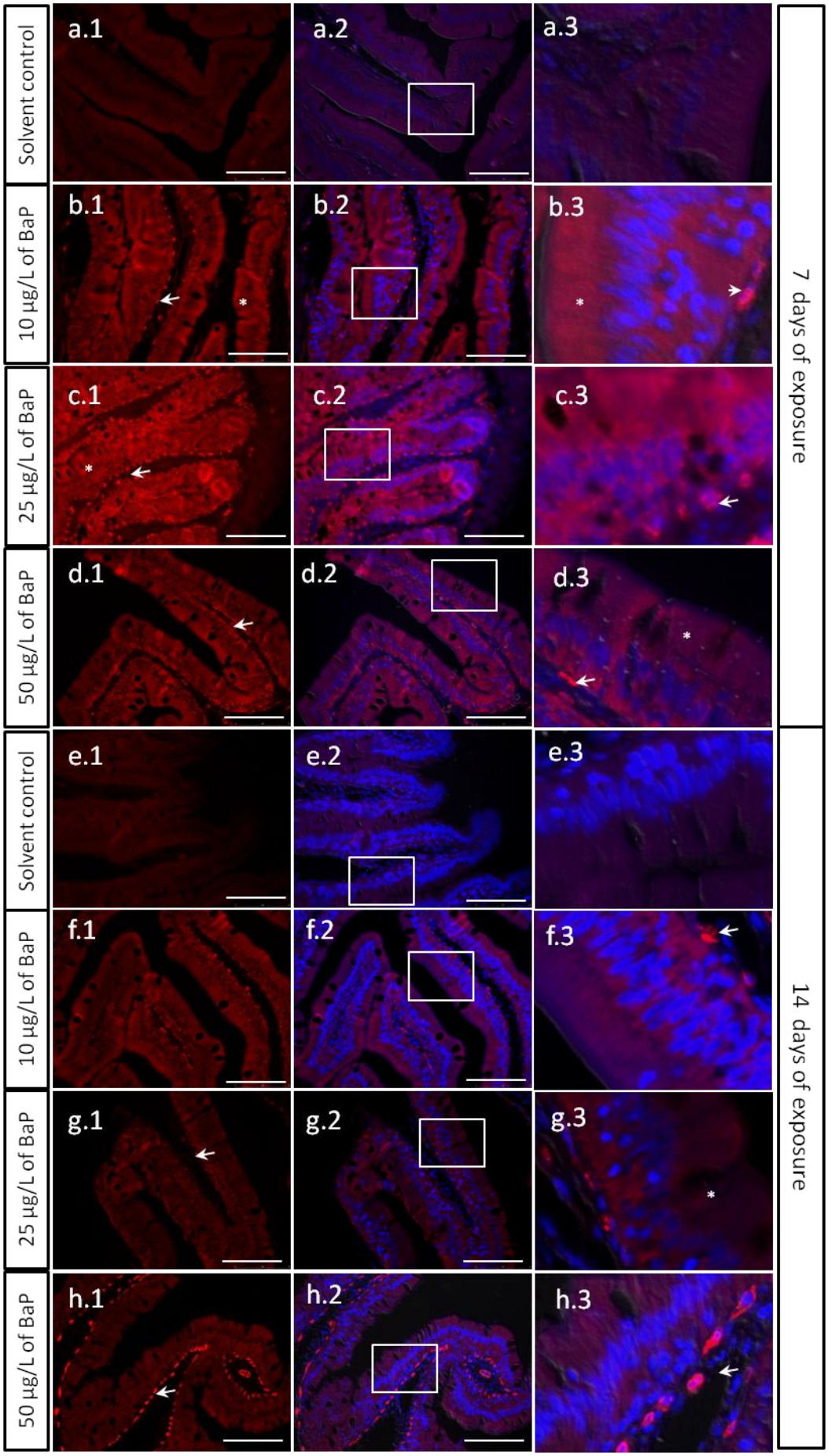
Solvent control
10 µg/L of BaP
25 µg/L of BaP
50 µg/L of BaP
7 days of exposure
Solvent control
10 µg/L of BaP
25 µg/L of BaP
50 µg/L of BaP
14 days of exposure
a.1
a.2
a.3
b.1
b.2
b.3
c.1
c.2
c.3
d.1
d.2
d.3
e.1
e.2
e.3
f.1
f.2
f.3
g.1
g.2
g.3
h.1
h.2
h.3

Figure 5.7 - Representative CYP1A immunostaining in proximal intestine of Nile tilapia exposed to BaP.

(a) 7 days of exposure, solvent control; (b) 7 days of exposure to 10µg/L of BaP; (c) 7 days of exposure to 25µg/L of BaP; (d) 7 days of exposure to 50µg/L of BaP; (e) 14 days of exposure, solvent control; (f) 14 days of exposure to 10µg/L of BaP; (g) 14 days of exposure to 25µg/L of BaP; (h) 14 days of exposure to 50µg/L of BaP. To provide structural information, blue nuclei were counterstained with DAPI and DIC image was overlaid (2), image (3) represents a higher magnification of square showed in (2). White arrows indicate positive labelling of intestinal vascular elements. (*) indicate positive labelling in enterocytes. Scale bar = 75µm.

5.5. Discussion

This study was conducted to further elucidate the presence of Pgp and CYP1A proteins in different tissues of the freshwater Nile tilapia, through the evaluation of protein levels and tissue distribution patterns after waterborne exposure to different BaP concentrations. The mammalian monoclonal antibodies C219 and C494, which recognize mammalian MDR1 gene product, were applied in Nile tilapia tissues with immunochemical approaches to detected Pgp (ABCB1), and different results were seen for each of the antibodies used. To our knowledge, antibodies raised against fish Pgp are not yet commercially available. Nevertheless, the C219 antibody, raised against mammalian Pgp, has been frequently used in immunochemical studies to determine the tissue distribution pattern of Pgp expression in a number of different aquatic species, such as worms (*Urechis caupo*, Toomey and Epel, 1993), mussels (*Mytilus californianus*, Cornwall at al., 1995) and fishes (Bard et al., 2002a; Bard et al., 2002b; Doi et al., 2001; Hemmer et al., 1995; Kleinow et al., 2000; Paetzold et al., 2009). This antibody recognizes an epitope common to both Pgp (ABCB1) and Spgp (ABCB11) (Childs et al., 1995; Georges et al., 1990; van den Elsen et al., 1999), hence it does not specify which ABCB protein is being labelled. Immunochemical results revealed that C219 reacted positively with a ~170kDa protein in liver samples of Nile tilapia (figure 5.1), and that the cellular localization of that protein was confined to hepatic bile canaliculi (table 5.1, figure 5.2b) of both solvent control and BaP exposed animals. Additionally, a very scarce positive reaction (1.35% of the samples analysed) was seen in apical surface of the enterocytes (table 5.1, figure 5.2f), and no staining was observed in gill after probing with C219 (figure 5.2d). Similar Pgp

staining patterns with the mAb C219 in liver and intestine were previously described for different fish species including high cockscomb blenny (*Anoplarchus purspurescens*; Bard et al., 2002b), several cyprinid fishes (Klobučar et al., 2010), catfish (*Ictalurus punctatus;* Doi et al., 2001), killifish (*Fundulus heteroclitus*; Bard et al., 2002a) and guppy (*Poecilia reticulata*; Hemmer et al., 1995), suggesting a positive reaction of C219 with Pgp in Nile tilapia. Nevertheless, we must also consider the possibility that C219 is also reacting with Spgp, rather than to Pgp alone in this study. In a previous work, we have found that neither Pgp (*ABCB1b*) or Spgp (*ABCB11*) genes were expressed in gills of Nile tilapia, moreover expression of Pgp in proximal intestine was higher than in liver, while Spgp showed higher mRNA expression in liver and lower expression in proximal intestine (Costa et al., 2012). Spgp is a bile-salt export pump predominantly, but not exclusively, found in the bile canaliculi of mammalian liver (Gerloff et al., 1998). This pattern is in accordance with our immunofluorescence and western blot results of C219, with an almost exclusive labelling in hepatic bile canaliculi, raising the possibility of a cross-reactivity of this antibody to Spgp in Nile tilapia.

The mammalian antibody C494 specifically recognizes an epitope in the C-terminal half of MDR1, and was shown to also cross-react with the mitochondrial enzyme Pyruvate Carboxylase (PC) (~130 kDa), on both immunoblots and immunohistochemical tissue sections of mammals (Rao et al., 1994). This antibody, unlike C219, showed a strong positive reaction in all the analysed tissues of Nile tilapia (liver, gills and proximal intestine), and covered a wider set of cellular types (figure 5.1, table 5.1). The hepatic and intestinal plasma membrane patterns of immunostaining seen in this study should represent true P-glycoprotein expression, given this is a transmembranar protein. Nevertheless, weak homogeneous, cytoplasmic or granular patterns of reactivity may represent staining of the mitochondrial PC cross-reactive epitope rather than positive staining for P-glycoprotein, in particular in liver and intestine, which are gluconeogenic tissues with proven PC activity (Böttger et al., 1969; Croset et al., 2001). Simultaneous positive labelling for C219 and C494 was seen in biliary canaliculi of liver and apical surface of the enterocytes, although the latter was much more frequent and intense after probing with C494. The mAb C494 has rarely been applied in fish tissues to detect the presence of Pgp. To our knowledge only Hemmer and coworkers (1995) have used this antibody in teleost fish, and also reported different tissue staining patterns in comparison to C219. In that study positive staining for both C219 and C494 was seen in intestine, kidney and exocrine pancreatic tissues. Tissues that stained positive for C219 but not C494 were bile canaliculi, pseudobranch and gill chondrocytes. Interrenal tissue, blood vessels and branchial transverse septum stained positive with C494, but not with C219.

Gin and coworkers (Ginn, 1996) have evaluated the cellular localization of Pgp in normal and neoplastic canine tissues with different antibodies (including C219 and C494), and indicated C494 as the antibody of choice for canine tissues based on quality of staining produced, specificity and economic advantages. In feline tissues, the cellular distribution pattern of Pgp was different when probing with C219 or C494, and the C494 staining pattern revealed a tissue distribution and cellular localization of Pgp similar to those found in human and canine tissues when using the same antibody (Van Der Heyden et al., 2009), and also similar to Nile tilapia liver and intestine staining patterns. As stated above, no Pgp (*ABCB1b*) mRNA expression was detected in Nile tilapia gill (Costa et al., 2012), which strongly contrasts with the results from this study, with labelling of the gill after probing with the mAb C494. The absence of Pgp mRNA expression in fish gills has also been described for other species, like trout (Loncar et al., 2010; Zaja et al., 2008). Nevertheless, Fischer and coworkers (2011) detected the presence of mRNA transcripts of two different Pgp isoforms in a permanent gill cell line of trout, and there is also immunochemical evidence of Pgp expression in gill of other aquatic species (Hemmer et al., 1995; Keppler and Ringwood, 2001a). In light of these results, and assuming the recently proposed possibility of the existence of more than one Pgp isoform in fish (Annilo et al., 2006; Fischer et al., 2011), it is possible that a different Pgp isoform is being detected in Nile tilapia gills in this study, when compared to our previous study (Costa et al., 2012). Although we have tried different testing conditions, including what is described in previous studies with the use of mAb C494 (Rao et al., 2004), we were unable to confirm the size of the protein that was reacting positively in Nile tilapia tissues to the mAb C494, as the antibody was not functional in the western blot technique. There is limited information on the use of this antibody in western blot, as the majority of the studies have been applying the mAb C494 only in immunohistochemical sections (Gin, 1996; Van Der Heyden et al., 2009, 2011). Nevertheless, tissue distribution pattern of reactive Pgp to the mAb C494 in Nile tilapia tissues, revealed similar results to those described in mammals and other fish species (Ginn, 1996; Hemmer et al., 1995; Van Der Heyden et al., 2009), with labelling of epithelial tissues involved in secretion, absorption or serving a barrier function, which is in agreement with the assumed role of Pgp in protection against xenobiotic insults (Szakács et al., 2008). Pgp expression, considering probing results with both mAb antibodies C219 and C494, was not altered in animals exposed to BaP, in comparison to solvent control. The question of whether or not BaP is a Pgp substrate remains in discussion, with some studies presenting controversial results in various animal models (Buesen et al., 2002; Chao Yeh et al., 1992; Colombo et al., 2003; Fardel et al., 1996; Schuetz et al., 1998; Zaja et al., 2011). Specifically in Nile tilapia, some doubts remained after analysing the mRNA expression of Pgp after exposure to BaP (Costa et al.,

2012), and the findings from this study indicate that BaP compound is not itself a substrate for Pgp.

Immunochemical results showed that the fish monoclonal antibody C10-7, directed against CYP1A protein, detected a ~55kDa protein in liver, gills and proximal intestine of Nile tilapia (figure 5.3). Additionally, the cellular distribution pattern of CYP1A (figures 5.5-5.7, table 5.2), is in agreement with those described in other fish studies (Bard et al., 2002b; Jönsson et al., 2006; Ortiz-Delgado et al., 2005; Van Veld et al., 1997). These results validate the use of this mAb to specifically detect CYP1A in Nile tilapia. Higher basal levels of CYP1A protein (i.e. non-exposed animals from solvent control group) were found in liver, followed by gill and proximal intestine. Additionally, a dose-dependent induction of CYP1A protein was observed in all tissues after exposure to BaP, with liver showing statistically significant differences in CYP1A protein of exposed animals in comparison to the control (figures 5.3 and 5.4). Maximum fold inductions of CYP1A were seen in proximal intestine (up to -176 fold), followed by liver and gill (up to -16 fold). Concordant results were seen with immunofluorescence analysis (table 5.2, figures 5.5 – 5.7). Moreover, these results are highly correlated with CYP1A-associated catalytic activity and *CYP1A* mRNA expression seen after BaP waterborne exposure in Nile tilapia tissues (Costa et al., 2011; Costa et al., 2012), validating the importance of extra-hepatic tissues (like gills and intestine) in the first-pass metabolism of waterborne pollutants. CYP1A induction is also in agreement with what was expected, considering the high affinity of these type of pollutants to bind the aryl-hydrocarbon receptor (AhR). AhR controls the expression of CYP450 enzymes, including CYP1A (Hankinson, 1995), and this response pattern has been described for other fish species (Jönsson et al., 2006; Ortiz-Delgado et al., 2008; Van Veld et al., 1997).

The hypothesis that Pgp and CYP1A share regulatory controls has been widely investigated in mammals (Buesen et al., 2002; Fardel et al., 1996; Lampen et al., 2004; Schuetz et al., 1998) and, to a lesser extent, in aquatic organisms (Bard et al., 2002b; Paetzold et al., 2009). In contrast to CYP1A, which is induced by AhR agonists, Pgp exhibits a more variable response, depending on the chemical and on the experimental model used. In Nile tilapia, CYP1A and Pgp showed different response patterns to the presence of BaP, suggesting that these two defence mechanisms do not share the same regulatory controls in this species. Nevertheless, these results point to the need to further elucidate the apparently complex regulatory mechanisms of Pgp, a putatively important protein in all organisms.

5.6. Conclusions

In conclusion, different tissue distribution patterns of Pgp in Nile tilapia were seen after probing with two different mammalian mAb, C219 and C494. Results for both antibodies follow the cellular distribution patterns described in previous studies, with C219 showing an almost exclusive labeling of the hepatic bile canaliculi, while the C494 staining pattern covered a wider set of cell types in liver, gills and proximal intestine. The mAb C10-7 was validated in this study to specifically detect CYP1A protein in Nile tilapia, and this protein was found to be expressed in liver, gill and proximal intestine of Nile tilapia. Results suggest that, while in Nile Tilapia BaP apparently is not a substrate for Pgp, CYP1A is involved in its metabolism in barrier tissues, such as liver, gills and intestine.

5.7. Acknowledgements

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Chapter 6

ABC transporters, CYP1A and GSTα gene transcription patterns in developing stages of the Nile tilapia (*Oreochromis niloticus*)

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6. ABC transporters, *CYP1A* and *GSTα* gene transcription patterns in developing stages of the Nile tilapia (*Oreochromis niloticus*)

6.1. Abstract

In fish, some ABC transporters are implicated in a multixenobiotic resistance (MXR) mechanism to deal with the presence of xenobiotics, by effluxing them, or their metabolites, from inside the cells. These efflux transporters have been considered an integral part of the cellular detoxification pathways, acting in coordination with phase I and II detoxification enzymes. However, the full characterization of this detoxification system is still incomplete, especially during the developmental stages of aquatic organisms, which are particularly sensitive periods to the presence of anthropogenic contamination. The goal of this study was to evaluate the mRNA expression dynamics of putatively important MXR proteins (ABCB1b, ABCB11, ABCC1, ABCC2 and ABCG2a) and phase I (CYP1A) and II (GSTα) biotransformation enzymes, during the embryonic and larval development of the specie *Oreochromis niloticus* (Nile tilapia). Our results showed that *ABCB1b, ABCC1*, *CYP1A* and *GSTα* transcripts are maternally transmitted. Transcripts for *ABCB11*, *ABCC2* and *ABCG2a* were only detected after the pharyngula period, which precedes a highly sensitive stage in embryonic development, the hatching. This study has shown, for the first time, very distinct expression patterns of genes encoding for proteins involved in protection mechanisms against pollutants during the development of Nile tilapia. Moreover, the temporal pattern of gene expression suggests that increased intrinsic protection levels are required at specific developmental stages.

Keywords: *MXR, Biotransformation enzymes, embryogenesis, Oreochromis niloticus, qRT-PCR*

6.2. Introduction

The aquatic environment is persistently loaded with complex mixtures of structurally different chemicals, resulting in the exposure of living organisms to these toxicants with consequent negative effects. Recent studies have indicated that the phenotype of multixenobiotic resistance (MXR), occurring in aquatic organisms, represents a general biological defense mechanism and may be responsible for their ability to deal with both endo- and xenobiotics (Bard, 2000). Some members of the ATP binding cassette (ABC) superfamily have been implicated in this mechanism, by acting as efflux pumps of a wide variety of toxicants and/or their metabolites from inside the cells in an ATP driven process (Epel, 1998; Kurelec, 1992). MXR is analogous to a previously described phenotype of multi-drug resistance (MDR), first observed in mammalian tumor cells (Gottesman et al., 1996), and related to the overexpression of an ABC transporter (ABCB1) responsible for the efflux of a high number of anti-cancer agents (Chan et al., 2004). As reviewed by Leslie and collaborators (2005) ABC proteins that confer multidrug resistance include, but are not limited to, P-glycoprotein (ABCB1), multidrug-resistance associated proteins 1 and 2 (ABCC1 and ABCC2) and the breast cancer resistance associated protein 2 (ABCG2). Additionally, the bile salt export pump (BSEP, ABCB11), responsible for the excretion of the highly toxic bile salts from hepatocytes into the bile has also been studied in aquatic organisms (Costa et al., 2012; Loncar et al., 2010; Zaja et al., 2008) due to its physiological function and also due to the high degree of similarity with ABCB1. These proteins have a broad range of substrates, and some specificity exists within each subfamily when it comes to transport mechanisms and chemical composition of substrates (Leslie et al., 2005). Based on the substrate specificity of each subfamily, these efflux transporters have been considered as an integral part of the cellular detoxification system, acting in a coordinated fashion with phase I and II biotransformation enzymes (Bard, 2000; Szakács et al., 2008; Xu et al., 2005). It is believed that ABCB1 acts as a first line of defense preventing unmodified compounds from accumulating in the cell (phase 0), while ABCCs and ABCG2 transport products of phase I and II metabolisms in the form of organic anions conjugated to glutathione, glucoronide sulphate or other polar groups, thus acting in phase III of cellular detoxification (Bard, 2000; Leslie et al., 2005; Paetzold et al., 2009). Although information regarding ABC transporters in aquatic organisms is still limited, these proteins have been identified in various species, and studies involving their response patterns to environmental pollutants have increased in the last years (Bard et al., 2002a; Bard et al., 2002b; Costa et al., 2012; Diaz de Cerio et al., 2012; Long et al., 2011b; Paetzold et al., 2009; Zucchi et al., 2010). Cytochrome P450 1A (CYP1A) and

Glutathione S-transferase α (GSTα) are important phase I and II detoxification enzymes that catalyze reactions of xenobiotic conversion (oxidation/reduction – phase I, conjugation – phase II) into a more water-soluble form, which can be more easily excreted from the cell than the parent compound (Lech and Vodicnik, 1985). The presence and function of these enzymes have been extensively studied and demonstrated in several animal species (Bilbao et al., 2010; Doi et al., 2004; Ferreira et al., 2008; Ferreira et al., 2010; Van Veld et al., 1997), including Nile tilapia (Costa et al., 2011; Costa et al., 2012). Recent studies have evaluated the simultaneous expression of ABC transporters and biotransformation enzymes in fish exposed to environmental pollutants (Costa et al., 2012; Della Torre et al., 2010; Paetzold et al., 2009; Zucchi et al., 2010), but the exact way of action of these important parts of cellular detoxification has not been fully demonstrated. Moreover, the vast majority of these studies have been conducted in juvenile or adult animals, and only a few have investigated the role of these proteins in the developing stages of aquatic organisms. The early life stages in fish development are particularly sensitive to anthropogenic contaminants (Buhl and Hamilton, 1991), implying that proteins involved in the protection of the organism, like ABC efflux transporters and biotransformation enzymes are of crucial importance during this period in all animals. High transcription and activity of efflux transporters have been reported in invertebrate larvae of aquatic species (Faria et al., 2011; McFadzen et al., 2000; Roepke et al., 2006; Toomey et al., 1996), but studies in vertebrate aquatic species, such as fish, are still very scarce (Long et al., 2011a; Long et al., 2011b).

Nile tilapia, *Oreochromis niloticus*, is one of the most important and useful species of cichlid fish, not only for fresh water aquaculture (FAO 2004), but also for research in a wide range of areas like toxicology (Almeida et al., 2001; Coimbra et al., 2007; Costa et al., 2011; Costa et al., 2012; Rey-Salgueiro et al., 2011), physiology (Wright and Land, 1998), endocrinology (Coimbra et al., 2005; Melamed et al., 1998), genomic biology and molecular genetics (Lee et al., 2005; McConnell et al., 2000; Santini and Bernardi, 2005).

The aim of this study was to evaluate the transcription patterns of genes involved in the detoxification pathways of aquatic organisms (*ABCB1*, *ABCB11*, *ABCC1*, *ABCC2*, *ABCG2*, *CYP1A* and *GSTα*) during embryonic and larval stages of *O. niloticus*, which are highly sensitive periods to the presence of toxicants in the environment.

6.3. Materials and methods

6.3.1. Biological material

Embryos used in the present work were obtained from breeders raised in the laboratory (CIIMAR, Porto, Portugal). Breeders stock (approximately 2 years old) were maintained in 500 L tanks (5-7 animals per tank), supplied with biological filtration and continuous aeration. During the non-reproductive season, dechlorinated tap water was used at a temperature of 20±2 ºC, with a 12 h:12 h (light:dark) photoperiod. Fish were fed commercial food pellets (Aquasoja, Portugal), until satiation, once a day.

6.3.2. Embryos collection and rearing conditions

Embryos and larvae used in this study were collected after natural female spawning and subsequent male fertilization of the eggs. Embryos from three different mothers were used in the study to reduce the variations in the rate of development previously seen among embryos of the same clutch, as well as among different clutches (Fujimura and Okada, 2007; Morrison et al., 2001). To induce reproduction, water temperature was gradually raised to 28±1 ºC. After approximately one week at high temperature, both males and females started to show clear signs of reproductive activity, including protuberant genital papilla in females, and the digging of circular holes in the substrate by dominant males. Animals were monitored during this period, and embryos were collected from female´s mouth immediately after fertilization. Embryos were separated into 5 L aquaria (250 eggs per aquarium) at 28±1 ºC and were allowed to develop with constant water renewal, and strong aeration to assure the continuous movement of the eggs. At different time points, embryos or larvae were collected and, after observation under a stereo microscope, those showing normal development were placed in RNAlater at 4 ºC overnight, and stored at -80 ºC until total RNA extraction.

Time points for embryo and larvae collection were chosen based on the developmental embryonic and larval stages of *O. niloticus* described by Fujimura and Okada (2007). Samples were collected in 18 embryonic stages (1-18), grouped in 7 periods (zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching), and 7 larval stages (19-25), grouped in 2 periods (early larvae and late larvae).

6.3.3. RNA isolation and cDNA synthesis

Total RNA from embryo and larvae was isolated from pools of the three different clutches of embryos composed by 4-6 six eggs and 2-4 larvae using the PureZOL RNA isolation reagent (Biorad), according to the manufacturer´s protocol. Quality of total RNA was assessed by separation in 1% agarose gel electrophoresis (in Tris-acetate-EDTA – TAE – buffer) stained with Gel Red (Biotium), and by the measurement of the ratio of the optical density at λ260/280 nm (1.8-2.0). RNA was quantified using Quant-IT RiboGreen RNA Reagent and Assay Kit (Invitrogen) using a Fluoroskan Ascent, Labsystems, to measure fluorescence at λexcitation 480nm and λemission 520nm. One microgram of total RNA was subjected to digestion of genomic DNA using Deoxyribonuclease I, Amplification Grade (Invitrogen), and first strand cDNA was synthesized using the iScript cDNA Synthesis Kit from BioRad, according to the manufacturer´s protocol.

6.3.4. Quantitative real-time PCR (qRT-PCR)

Gene expression of *ABCB1b* (Annilo et al., 2006; in www.ensemble.org *ABCB1b* is annotated as *ABCB4*), *ABCB11*, *ABCC1*, *ABCC2*, *ABCG2a*, *CYP1A* and ***GSTα*** was assessed in embryo and larvae at the different time points of development, by means of quantitative real time PCR (qRT-PCR). Specific primers for *ABCB1b*, *ABCB11***,** *ABCC1***,** *ABCC2*, *ABCG2a*, *CYP1A***,** ***GSTα*** and *18S rRNA* were used according to Costa et al. (2012). Identities of the amplicons for qRT-PCR were confirmed by cloning and sequencing of the DNA fragments as described in Costa et al. (2012). Primer sequences, amplicon lengths, qRT-PCR efficiencies and Genbank accession numbers of target sequences are given in table 6.1. Reactions for qRT-PCR were conducted in a IQ5 BioRad, with 10 µl of SYBR Green Supermix (BioRad), 2 µl of each primer (6 µM) and 1 µl of cDNA, in a total volume of 20 µl. Conditions were as follows: 95 ºC for 3 min, followed by 40 cycles of 95 ºC for 10 s, 54 ºC for 30 s and 72 ºC for 30 s. At the end of each run a melting curve analysis was done (from 55 to 95 ºC) to determine the formation of the specific products. Samples were run in duplicate. No template controls were run to exclude contamination and the formation of primer dimers. To determine the efficiency of the PCR reactions (table 6.1), standard curves were made for all genes, with 6 serial dilutions of the template (concentrations range from 0.05 to 50 ng/µl), and the slopes and regression curves were calculated.

Quantification of the mRNA expression of the genes in study, *ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2*, *ABCG2a*, *CYP1A* and *GSTα*, during the embryonic and larvae development of *O. niloticus* was performed by normalization against the housekeeping gene (*18S rRNA*), by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), since efficiency of the PCR reactions was close to 100% (table 6.1). The mean mRNA expression of the different stages/periods was calculated based on the samples showing detectable expression. *18S rRNA*, *Glyceraldehyde 3-phosphate dehydrogenase* (*GADPH*), *Elongation factor 1* (*EF1*), *Ribossomal protein L17* and *ß-actin* were evaluated as possible reference genes, and *18S rRNA* was chosen, since it has been shown to be the most stable gene at the analyzed developmental stages (data not shown). Even though *18S rRNA* showed to be the most stable gene throughout the development, it was differently expressed from cleavage to blastula and from gastrula to segmentation, being stable in three clustered groups during the stages that were analyzed in this study. Thus, the final data will be presented in separated graphs following the stability of the housekeeping gene (zygote to cleavage; blastula to gastrula and segmentation to late larvae). For this reason, no comparison of the gene transcription data from cleavage to blastula or from gastrula to segmentation will be performed.

6.3.5. Statistical analysis

Differences between gene expression in the different time points of embryonic and larvae development were evaluated by means of a one-way ANOVA, followed by a multiple comparison test (Tukeys test) at a 5% significance level. Some data had to be log or square root transformed in order to fit ANOVA assumptions. All tests were performed using the software Statistica 7 (Statsoft, Inc., 2001).

6.3.6. Ethics statement

The animals used in the research described in this paper were treated in accordance with the Portuguese Animals and Welfare Law (Decreto-Lei nº 197/96) approved by the Portuguese Parliament in 1996. Institutional animal approval by CIIMAR/UP and Direcção Geral de Veterinária was granted for this study.

Table 6.1 – Primer sequences, amplicon lengths and efficiency of reaction, used in ABC transporters, *CYP1A*, *GSTα* and *18S rRNA* gene expression quantification by qRT-PCR in Nile tilapia

Target gene	Sense	Antisense	Amplicon length	Efficiency of the PCR reaction	Genbank ID
ABCB1b	cgttcctcaaggtgatggct	ggctgcattgcaccattgat	91 pb	98.5%	**GQ911571**
ABCB11	ctggtcagacactggccttt	caggaaagacacgttgacgc	143 pb	110.0%	**GQ911570**
ABCC1	atccgtgagagtgaccag	caaatgacacaatgaagtttcc	117 pb	99.7%	**GQ911567**
ABCC2	cctggttggcttgtctatatcc	ctcgctgtattcactcactctc	123 pb	107.6%	**GQ911569**
ABCG2a	tcatgaagccgggtctcaac	agacctgcagggtcctttct	96 pb	103.9%	**GQ911568**
CYP1A	cgtcgtcgtctctgttgcc	catcgtcgtggtggtcatagc	70 pb	96.6%	**GI13365614**
GSTα	aaatggatggcatgaagctc	tcgttctttgggatcctttg	92 pb	109.8%	**EU234530**
18S rRNA	cggaaggatcattactggctacac	agaccctcggcggcaaag	78 pb	100.1%	**DQ397879**

6.4. Results

Results for the mRNA expression during zygote and cleavage periods of the genes evaluated in this study are displayed in figure 6.1. Only *ABCB1b*, *ABCC1*, *CYP1A* and *GSTα* showed to be expressed since the initial periods of embryonic development of *O. niloticus*, zygote and cleavage. According to Fujimura and Okada (2007), in *O. niloticus* embryo development, these periods can be divided into five stages (1 to 5) that comprise the first 4 hours post fertilization (hpf). Gene expression showed a downward trend from stage 1 (zygote) to stage 2 (first cleavage stage), followed by an increase after stage 3. This pattern was clear for *ABCC1* mRNA expression (figure 6.1b), where no mRNA was detected in samples from stage 2, and only 25 and 50% of the samples from stages 3 and 4, respectively, showed *ABCC1* mRNA expression. Moreover, gene expression in final cleavage stages (4 and 5) was significantly higher than in earlier stages (1 to 3). Regarding *CYP1A*, although no significant differences were seen in mRNA expression during zygote and cleavage periods (figure 6.1c), mRNA expression was only detected in 50% of stage 2 and 75% of stage 3 samples. Gene expression of *ABCB1b* (figure 6.1a) and *GSTα* (figure 6.1d) was detected in all samples during the first five stages of *O. niloticus* development. Additionally, *ABCB1b* and *GSTα* were the most expressed genes in these stages, followed by *ABCC1* and *CYP1A*.

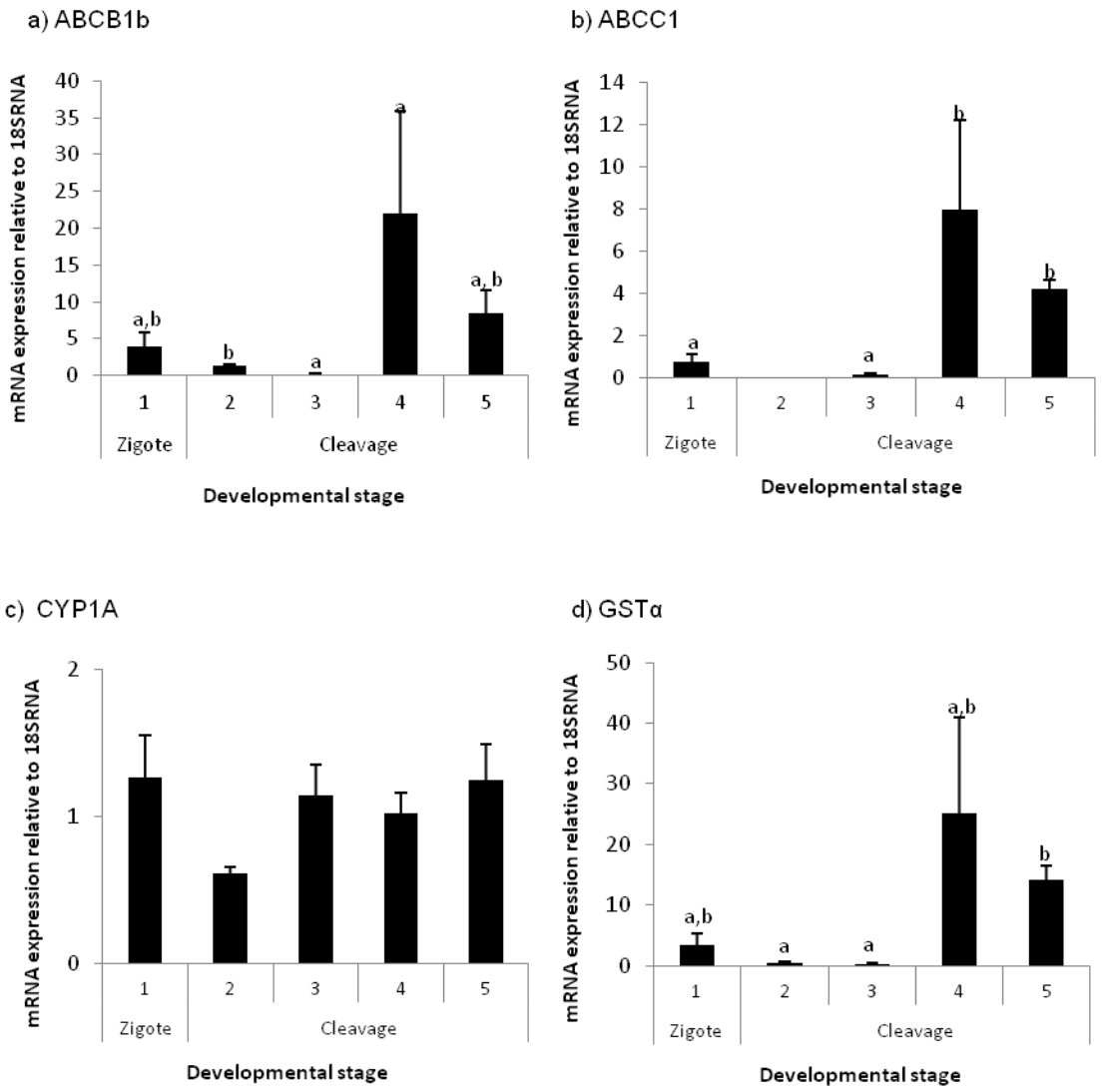

Figure 6.1 – Relative mRNA expression of *ABCB1b* (a), *ABCC1* (b), *CYP1A* (c) and *GSTα* (d) during the first five stages (1-5) of embryonic development in *O. niloticus* comprising the zygote and cleavage periods.

Expression was measured by qRT-PCR and quantified by normalization against the housekeeping gene (*18S rRNA*) by the $2^{-\Delta\Delta Ct}$ method. Results are given as mean±SE (n = 4). Calculated mean include samples with detectable mRNA expression only. Different letters denote significant differences ($p<0.05$) between stages.

In blastula (4-22 hpf) and gastrula (22-26 hpf) stages, only mRNA expression of *ABCB1b*, *ABCC1*, *CYP1A* and *GSTα* was detected, as for zygote and cleavage. According to the staging system described by Fujimura and Okada (2007), each of these periods can be subdivided in 2 stages (6 and 7 for blastula, 8 and 9 for gastrula). Since no significant differences were seen in mRNA expression between the stages of each period, data from stages 6 and 7 was grouped in blastula, and data from stages 8 and 9 was grouped in gastrula and results are presented in figure 6.2. No significant differences were seen between mRNA transcriptions of the two periods for any of the genes, although a tendency for an increase from blastula to gastrula was observed. Moreover, all genes detected at these stages showed similar levels of expression.

Gene expression of *ABCB1b*, *ABCC1*, *CYP1A* and *GSTα* from segmentation (26 to 30 hpf) to late larvae (11 to 13 days post fertilization – dpf) are displayed in figure 6.3. Data from stages belonging to the same period of development were grouped together (according to Fujimura and Okada, 2007). *ABCB1b* and *CYP1A* mRNA expression decreased after the segmentation period. *ABCB1b* mRNA levels at segmentation were significantly higher than the following stages (figure 6.3a), while *CYP1A* mRNA showed a pattern of decreasing expression until late larvae (figure 6.3c). A different scenario was seen for *GSTα*, with a significant increase in mRNA after the hatching period that was maintained until the end of the late larvae period (figure 6.3d). *ABCC1* mRNA expression showed no significant changes from segmentation to late larvae (figure 6.3b). *ABCB1b* mRNA levels were higher than *ABCC1*, *CYP1A* and *GSTα* in the segmentation period, but decreased to similar levels in the following stages.

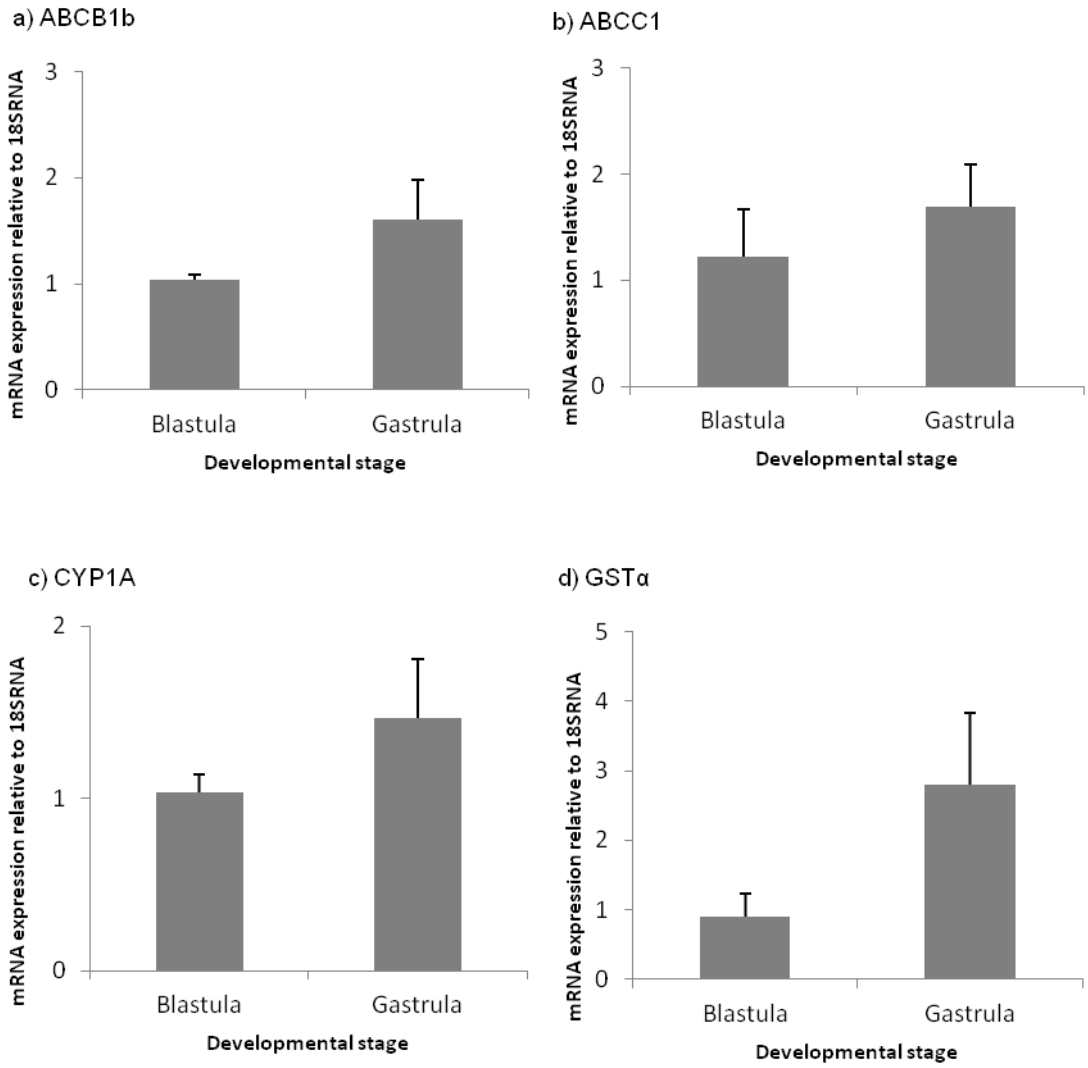

Figure 6.2 - Relative mRNA expression of *ABCB1b* (a), *ABCC1* (b), *CYP1A* (c) and *GSTα* (d) during the blastula and gastrula periods of embryonic development in *O. niloticus*.

Expression was measured by qRT-PCR and quantified by normalization against the housekeeping gene (*18S rRNA*) by the $2^{-\Delta\Delta Ct}$ method. Results are given as mean±SE (n = 4-5).

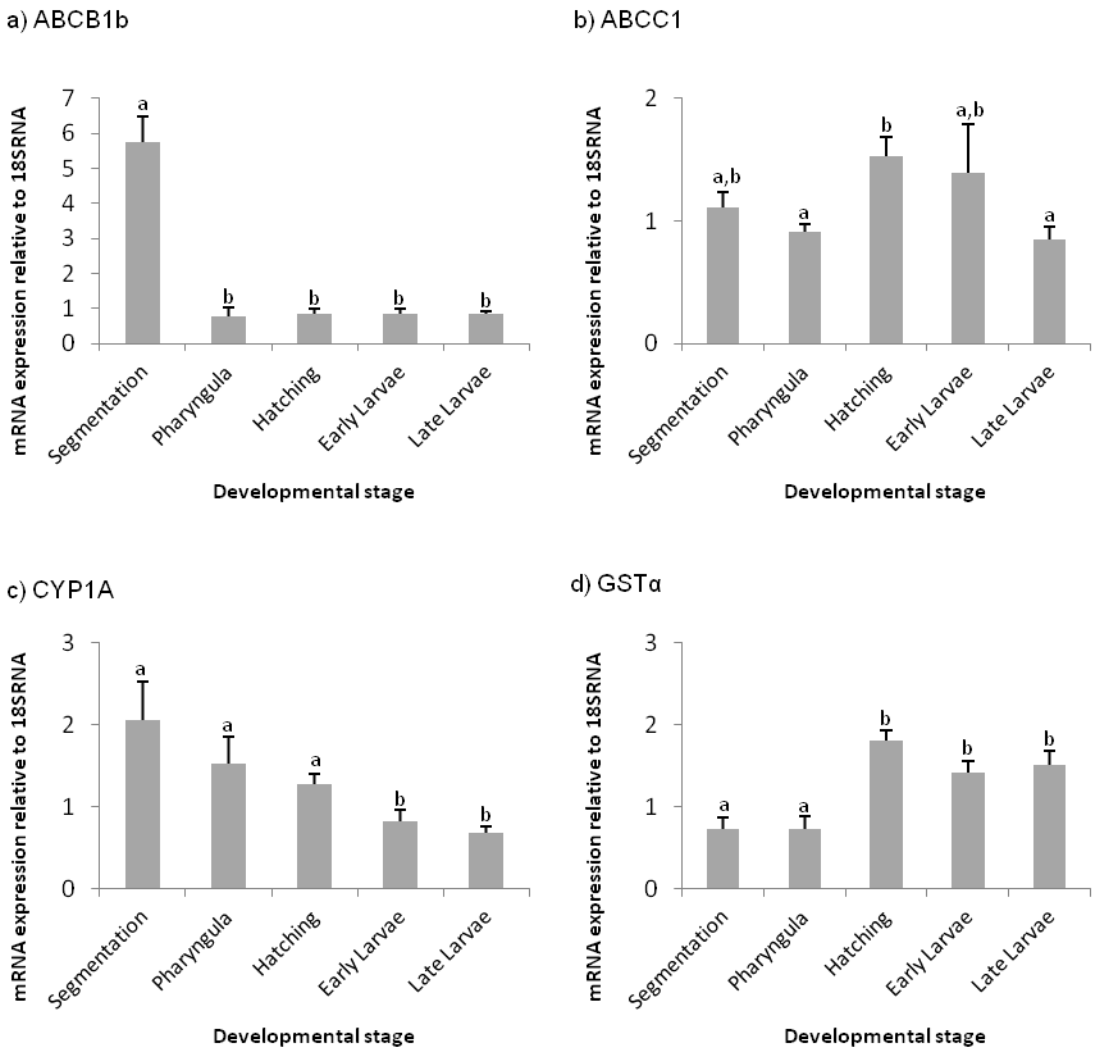

Figure 6.3 - Relative mRNA expression of *ABCB1b* (a), *ABCC1* (b), *CYP1A* (c) and *GSTα* (d) during the last embryonic (segmentation to hatching) and larval periods (early and late larvae) in *O. niloticus* development.

Expression was measured by qRT-PCR and quantified by normalization against the housekeeping gene (*18S rRNA*) by the $2^{-\Delta\Delta Ct}$ method. Results are given as mean±SE (n = 8-12). Different letters denote significant differences ($p<0.05$) between stages.

In figure 6.4, mRNA expression of *ABCB11*, *ABCC2* and *ABCG2a* during embryonic and larval developmental stages in *O. niloticus* is displayed. Gene expression was only detected in pharyngula and following developmental periods, with a pattern of increasing mRNA expression. This pattern was particularly clear for *ABCB11* (figure 6.4a) and *ABCC2* (figure 6.4b) where a significant increase ($p<0.05$) was seen after the pharyngula period. Similar levels of mRNA were seen in *ABCB11*, *ABCC2* and *ABCG2a* from pharyngula to late larvae. Main results obtained in this study are summarized in figure 6.5.

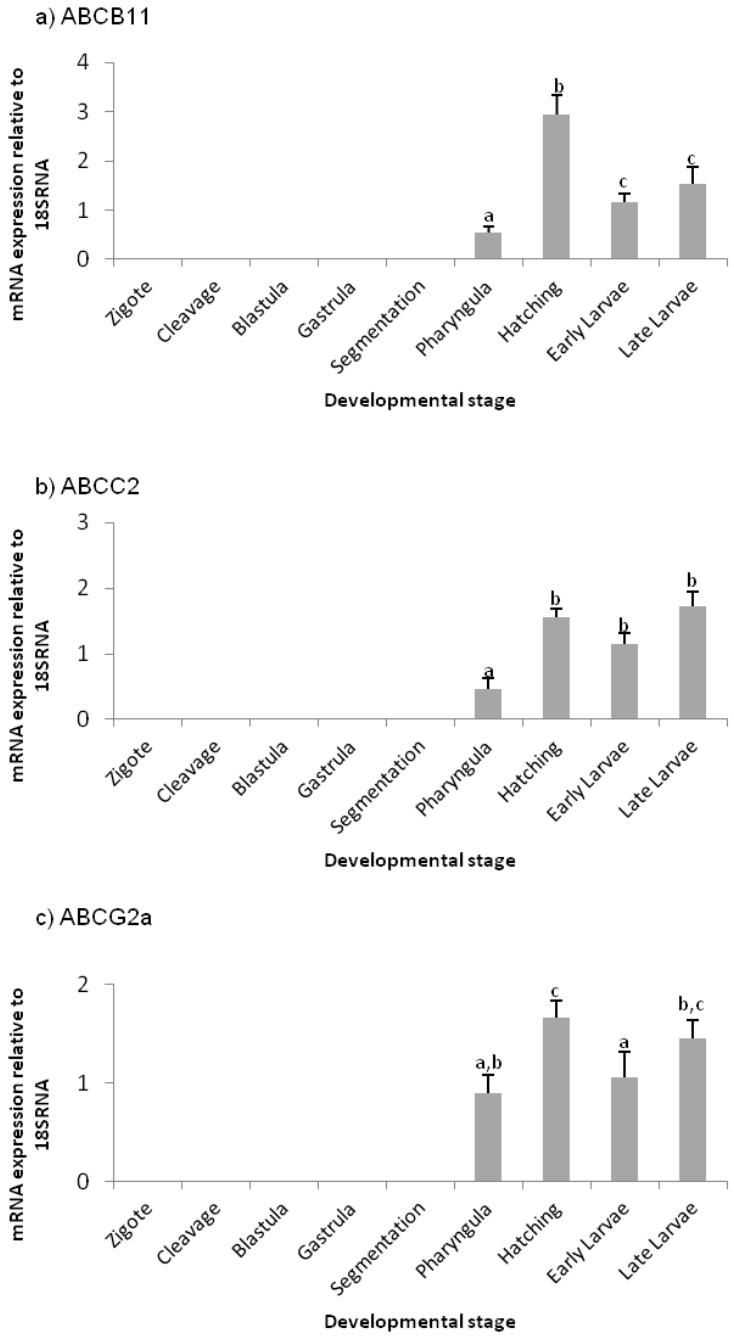

Figure 6.4 – Relative mRNA expression of *ABCB11* (a), *ABCC2* (b) and *ABCG2a* (c) during the embryonic and larval periods (zygote to late larvae) in *O. niloticus* development.

Expression was measured by qRT-PCR and quantified by normalization against the housekeeping gene (*18S rRNA*) by the $2^{-\Delta\Delta Ct}$ method. Results are given as mean±SE (n = 5-12). Different letters denote significant differences ($p<0.05$) between periods.

6.5. Discussion

Several studies have addressed the hypothesis of a coordinated regulation of MXR proteins and biotransformation enzymes in aquatic organisms, resulting in the activation of an important mechanism of cellular protection against xenobiotic insults (Bard, 2000; Costa et al., 2012; Leslie et al., 2005; Xu et al., 2005). Although the presence and functionality of these proteins have been demonstrated in several aquatic species (Diaz de Cerio et al., 2012; Fischer et al., 2011; Loncar et al., 2010; Paetzold et al., 2009; Zaja et al., 2008), the full characterization of this mechanism is not yet complete mainly during the early life stages of fish development, which is a particularly sensitive period to the presence of contaminants (Buhl and Hamilton, 1991). In this study, we analyzed the gene transcription patterns of several ABC transporters (*ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2* and *ABCG2a*) and phase I (*CYP1A*) and II (*GSTα*) biotransformation enzymes, during the embryonic and larval stages of *O. niloticus*.

Only *ABCB1b*, *ABCC1*, *CYP1A* and *GSTα* mRNA were expressed since the onset of development of the embryo (figures 6.1 and 6.5). *ABCB11*, *ABCC2* and *ABCG2a* mRNA expression was only detected after the pharyngula period (figures 6.4 and 6.5). In mammals, murine oocytes and early embryos express *ABCB1* (Elbling et al., 1993) and in porcine oocytes both *ABCB1* and *ABCC1* mRNA expression was detected (Takebayashi et al., 2001). In the early stages of embryonic development of aquatic invertebrate species, proteins associated with MXR mechanism were shown to play an active role in embryos protection. *ABCB1* expression was detected by western blot in *Urechis caupo* embryos since 2-cell stage, and a biodegraded crude oil fraction (BWSF) was non-toxic to those embryos/larvae, while in *Lytechinus anemesus* embryos, where no MXR efflux capacity was observed, exposure to BWSF caused developmental abnormalities (Hamdoun et al., 2002). In zebra mussel, *ABCB1* mRNA was detected in eggs, although MXR mediated efflux capacity only occurred in one day old larvae (Faria et al., 2011). In fish developmental stages ABC transporters characterization is still very limited. However, zebrafish transcripts of *ABCC1* gene were detectable in four-cell stage embryos (1hpf), indicating that this gene is maternally expressed, and mRNA expression was significantly induced by toxic heavy metals (Long et al., 2011a). In other fish species, CYP1A mRNA and/or activity was also found to be present and inducible by the exposure to contaminants in later developmental stages, like hatching (Engwall et al., 1994; Goksøyr et al., 1991; Hodgson and George, 1998; Peters et al., 1996; Sarasquete et al., 2001). Nevertheless, CYP1A activity was already inducible by the presence of PCBs in killifish pre-hatched embryos (Binder et al., 1985), and the knockdown of *CYP1A* mRNA enhanced the

frequency of developmental disorders in zebrafish 26 hpf pre-hatched embryos exposed to 3,4-dichloroaniline, suggesting that *CYP1A* is translated into active protein in pre-hatching stages (Voelker et al., 2008). Similarly, the presence of the mRNA for phase II enzyme *GSTα* was previously described in plaice eggs (6 hpf) (Hodgson and George, 1998) and in early rice fish embryos (1 hpf) (Wu et al., 2011). Our results are in agreement with these studies, indicating that also in *O. niloticus* early embryos *ABCB1b*, *ABCC1*, *CYP1A* and *GSTα* mRNA transcripts are present, and are of maternal origin, suggesting a crucial role in these early stages of development, of these particular proteins. The mRNA expression of these maternally transmitted genes has decreased from zygote to cleavage first stage (figure 6.1). This pattern reflects the natural processes occurring in the embryos of maternal to zygote transition (MZT) (Tadros and Lipshitz, 2009). When this process is triggered, a subset of the maternal mRNAs is eliminated by means of maternally encoded products, and the transcription of the zygote genome begins, leading to the production of transcriptional activators that enhance the efficiency of zygote transcription, including proteins and microRNAs (miRNAs) that provide feedback to enhance the maternal mRNA degradation (Tadros and Lipshitz, 2009). Thus, we propose that degradation of maternal transcripts, in Nile tilapia, is occurring in stages 2 and 3, followed by the beginning of zygote transcription in stages 4 and 5 (figure 6.1). Blastula and gastrula periods follow the early mitotic cycles, and roughly coincide with the major wave of zygote genome activation in fish (Kane and Kimmel, 1993), generally resulting in the rise of mRNA transcripts of most genes. In our study, *18S rRNA* transcripts increased significantly from the cleavage to blastula periods (data not shown), precluding the comparison of mRNA expression transcripts between these periods. Genes that were found to be expressed since the zygote period were also detected during the blastula and gastrula periods (figure 6.2), being an indication that transcripts are being produced by zygote genome at this point. Characteristic processes occurring during the segmentation period in fish include the development of the somites, appearance of the rudiments of the primary organs, the beginning of morphological cell differentiation and the first movements of the body (Fujimura and Okada, 2007; Kimmel et al., 1995). Afterwards, the embryo enters the pharyngula period with a well developed notochord, and a newly completed set of somites that extend to the end of a long post-anal tail (Fujimura and Okada, 2007; Kimmel et al., 1995). These set of events might explain the decrease in mRNA expression of *ABCB1b* and *CYP1A* after the segmentation period (figure 6.3a and 6.3c). At the beginning of the organogenesis mRNA expression of these genes could be confined to some specific organs, that function as physiological/pharmacological barriers, as previously shown in tissues of adult fishes (Loncar et al., 2010; Sarasquete and Segner, 2000; Zaja et al., 2008). In particular, in Nile tilapia it was shown that *ABCB1b* is not expressed in the gill,

being more expressed in the liver and intestine, while *CYP1A* mRNA expression was much higher in the liver and gill, than in the proximal intestine (Costa et al., 2012). In contrast, *ABCC1* and *GSTα* seem to be more ubiquitously expressed in adult fish tissues (Costa et al., 2012; Kim et al., 2010; Li et al., 2010; Long et al., 2011a), which can reflect the mRNA transcription patterns seen in the late embryonic and larval stages described in this study (figures 6.3b and 6.3d). Additionally, besides its role as a phase II enzyme, GSTα is also an antioxidant enzyme, protecting the cells against reactive oxygen species (reviewed in van der Oost et al., 2003). A previous work has reported an increase in oxidative stress during the embryogenesis of Japanese rice fish (until hatching), accompanied by an increase in the mRNA expression of *GST1α* (Wu et al., 2011). Our results are in agreement with this work, and the increase in *GSTα* mRNA expression after the pharyngula period could be a physiological response to maintain oxidative stress balance, important for the normal development of the embryos. The pharyngula period was marked by the appearance of the first mRNA transcripts for the ABC transporters *ABCB11*, *ABCC2* and *ABCG2a* in Nile tilapia (figures 6.4 and 6.5), and in the hatching period mRNA expression increased significantly for these three genes accompanied with *GSTα* (figure 6.3d). During the hatching period the embryo continues to grow, opening of the mouth occurs and the morphogenesis of many of the rudiment organs, like the gills, is completed and slows down considerably, with some exceptions like the gut and associated organs (Fujimura and Okada, 2007; Kimmel et al., 1995). Moreover, the hatching of the embryos, a critical period during the development of many organisms, implies the loss of their protective “shell”, and consequently means that they become more exposed to the surrounding environment. The severity of this period may require an increase in the intrinsic defense mechanisms, which we believe is reflected by the increase of the mRNA expression of the majority of the genes evaluated in this study, *ABCC1*, *ABCB11*, *ABCC2*, *ABCG2a* and *GSTα* (figures 6.3 and 6.4). Previous works have reported an increase in the MXR mechanism after the hatching period in several invertebrate aquatic species (Faria et al., 2011; McFadzen et al., 2000; Minier et al., 2002). Moreover, similarly to what we found in Nile tilapia, *ABCC2* gene in zebrafish was only detected in 72hpf embryos (right before hatching) and the overexpression of zebrafish *ABCC2* in embryos decreased the cellular accumulation of heavy metals, suggesting an active role of ABCC2 protein in this stage (Long et al., 2011b).

In conclusion, the expression patterns of genes encoding for some of the most important proteins involved in mechanisms of protection against pollutants, like MXR proteins (ABCB1b, ABCB11, ABCC1, ABCC2 and ABCG2) and biotransformation enzymes (CYP1A and GSTα), were described in embryonic and larval developmental stages of a teleost fish, Nile tilapia, for the first time. Overall, this work has shown that

these genes present different expression patterns during the embryogenesis in Nile tilapia. While some of these genes are present since the early stages of development (*ABCB1b*, *ABCC1*, *CYP1A* and *GSTα*), others are only transcribed in later embryonic stages preceding sensitive periods of development (*ABCC2* and *ABCG2*), when the demand for these types of proteins may rise in order to assure the necessary levels of protection against toxicants from the surrounding environment (figure 6.5).

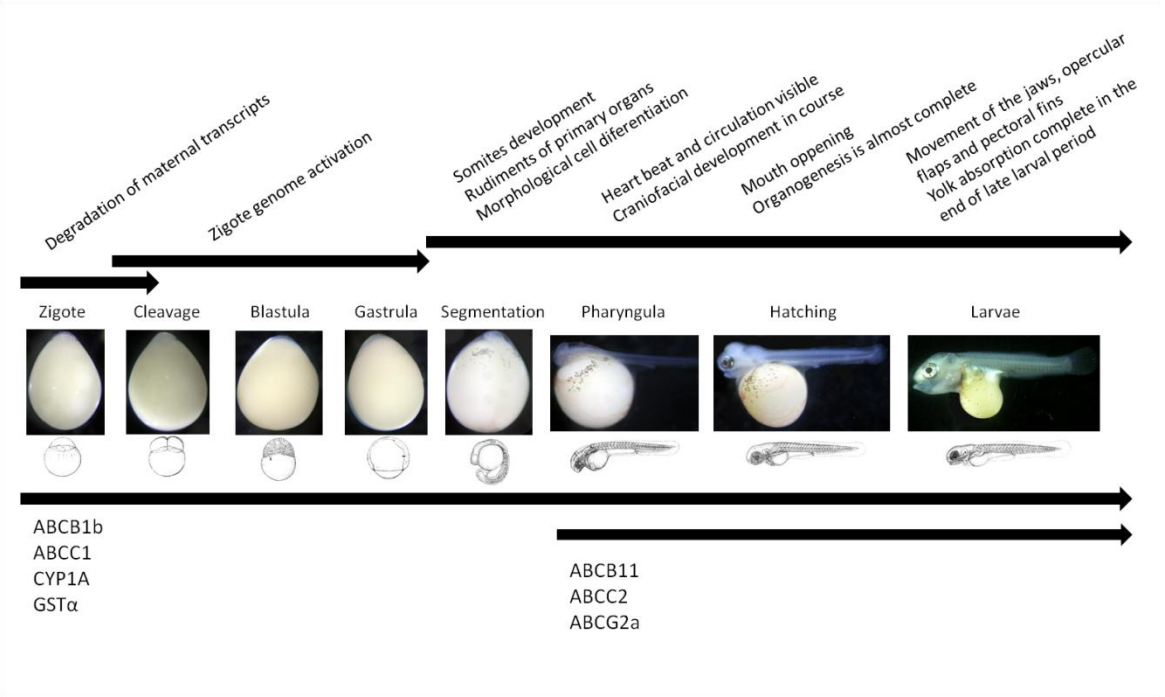

Figure 6.5 – Schematic representation of the main results achieved in this study, showing the temporal pattern of mRNA expression for the genes in study (*ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2*, *ABCG2a*, *CYP1A* and *GSTα*) in Nile tilapia developmental stages. Adapted from Kimmel et al. (1995) and Fujimura and Okada (2007).

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Chapter 7

Final remarks

7.1 General discussion

7.2 Conclusions

7.3 Future perspectives

7. Final remarks

7.1. General discussion

In the last decades, aquatic pollution, mainly resultant from the industrialization of the northern hemisphere, has been recognized as a global environmental problem, jeopardizing the survival rates of many species inhabiting the aquatic environments. Thus, the understanding of the subjacent parameters of cellular detoxification is an uppermost need, as it can provide environmentalists with crucial information that may allow a sustainable future for the environment. The biotransformation of toxic compounds into more easily excreted metabolites by phase I and phase II enzymes is one of the most relevant mechanisms adopted by living organisms, in order to cope with the presence of pollutants in their habitats. Recently, it has been proposed that some ABC efflux transporters can also be involved in the removal of parent or metabolized xenobiotics from the inside of the cells, in an ATP driving process, acting in a coordinated fashion with the biotransformation enzymes. The relevance of ABC efflux transporters was first recognized in humans, and they were found to be responsible for a MDR phenomenon by diminishing the efficacy of cancer treatments. Therefore, research that follows the clinical aspect of the ABC transporters mode of action is mostly focused on understanding how to block their activity, in order to assure the effectiveness of administered drugs in chemotherapeutic treatments. Being highly conserved through vertebrate species, the role of ABC transporters as key players in cellular detoxification was later recognized. In aquatic organisms, they are believed to protect the cells from pollutants in a mechanism named as MXR and, in contrast to the clinical aims, environmentalists' goals are to ensure that these transporters are operating optimally, hence continuing to keep toxicants out of the cells. Therefore, the full knowledge on the functionality of this cellular defence mechanism is of sovereign value both for environmental as for human health. Nevertheless, although there are increasing evidence for the presence of ABC efflux transporters in fish species at the genetic, immunochemical and functional levels, the knowledge of the role of these proteins is still rudimentary. Moreover, when addressing cellular detoxification systems, the bulk of the research has been done mainly in xenobiotic biotransformation and, to our knowledge, no *in vivo* studies have been directed to better acquaint the integration and cooperation of these efflux transporters with biotransformation enzymes in the detoxification pathway. In order to fill these gaps, this study was outlined to assess, for the first time, the response and the cooperation of the three phases of detoxification. To fulfil our goals, *in vivo* assays

of exposure of Nile tilapia to a highly toxic PAH, BaP, were conducted using different routes of exposure. Subsequently, ABC efflux transporters and biotransformation enzymes responses to the presence of this xenobiotic were evaluated in different barrier tissues of Nile tilapia.

The biotransformation pathway of BaP was characterized by assessing the catalytic activities of phase I (EROD) and phase II (GSTs) biotransformation enzymes (chapter 2), after waterborne and dietary exposures, in different tissues of Nile tilapia. The biotransformation pathway was shown to be dependent on the route of exposure to the contaminant and different tissues were involved in BaP metabolism. Waterborne exposure led to BaP metabolism in liver, and also in the extra-hepatic tissues, gills and intestine, evidenced by a dose-dependent up-regulation of CYP1A-associated catalytic activity in these tissues. A different scenario was observed after the dietary exposure to BaP, where the induction of intestinal, but not hepatic EROD activity, pointed out that intestinal metabolism was the major detoxification pathway adopted by Nile tilapia. Together with the high correlations found between the levels of biliary BaP metabolites and EROD activity in all tissues, after both exposure routes, our results showed that liver, gills and intestine display a substantial role in the first-pass metabolism of environmental contaminants. Moreover, the data indicated that, after CYP1A metabolism, metabolites formed in extra-hepatic tissues can be reabsorbed into the blood for enteropatic circulation and then released in the gall bladder, as suggested for other species (Kleinow et al., 1998). EROD activity and biliary BaP metabolites reflected properly the contamination levels that fish were exposed to, which strongly support their use as biomarkers of exposure to PAHs. Moreover, the tissue-specific differences in EROD activity and the different levels of BaP metabolites, measured according to the type of exposure, can provide useful indications of the main exposure route to the contaminants in the field. Thus, the use of these biomarkers of exposure in extra-hepatic tissues should be proposed as a tool to use in environmental risk assessment monitoring studies, as they can provide additional information on the characterization of the type of exposure that animals are facing in the environment.

No relevant changes were observed in the activity of phase II biotransformation enzymes GST, in any of the tissues. In the literature, the use of GST activity as a biomarker of exposure to PAHs is controversial, with some authors reporting up-regulations, while others did not observed any changes or even reported reductions in GST activity (van der Oost et al., 2003). Nevertheless, the different GST isoforms respond differently after xenobiotic exposure in fish (Martínez-Lara et al., 1996; Perez-Lopez et al., 2002), which can indicate that the net result, measured by total GST activity, could be null

and not necessarily mean that GSTs were not involved in BaP metabolism. Even so, our results point to a low sensitivity of these enzymes as a phase II biomarker in Nile tilapia, as previously shown in other studies conducted in fish (Della Torre et al., 2010; Schreiber et al., 2006). For these reasons, more investment should be paid to the research of the specific isoforms that can be involved in xenobiotic metabolism.

One important feature of BaP is the highly reactive and toxic metabolites that can be formed after enzymatic biotransformation. One of BaP metabolites, the 3-OH-BaP in the free form, is known to be toxic with the ability to bind to DNA. By analysis of the FACs in bile, the presence of BaP metabolites, which includes phase I (free) and phase II (conjugated) metabolites, was shown (chapter 2). By HPLC analysis we were able to detect the presence of the free (toxic) and conjugated 3-OH-BaP metabolites in bile and plasma of BaP water exposed Nile tilapia (chapter 3). This study has further demonstrated the presence of a well-developed biotransformation system in this species, since no parent compound was detected, and the metabolite, 3-OH-BaP, was present, in free and conjugated forms, in bile and in plasma of exposed animals. Moreover, gill and intestinal EROD activities of waterborne exposed animals were positively correlated with free 3-OH-BaP in bile and total 3-OH-BaP in plasma, strengthening the involvement of extra-hepatic tissues in phase I BaP metabolism, and the release of metabolites formed in extra-hepatic tissues into the blood stream. The presence of the toxic form of 3-OH-BaP in plasma is of special concern, since this metabolite has been reported to have estrogenic activity and the ability to bind to macromolecules, such as DNA and proteins (hemoglobin) (van Lipzig et al. 2005, Sugihara and James 2003). Nevertheless, Nile tilapia has also shown to have the ability to neutralize the toxic phase I metabolite (free 3-OH-BaP) into non-toxic forms (conjugated forms) demonstrated by the predominance of conjugated over free metabolites in bile of waterborne exposed fish, that indicates the important role of phase II enzymes (GST, UGT and SULT). However, and despite the high G-3-OH-BaP levels detected in bile, no significant induction of UGTs activity was observed in Nile tilapia. UGTs, as GSTs, exhibit a broad specificity for structurally diverse compounds and different UGTs isoforms have been described in fish (George, 1994), Thus, the measurement of UGTs catalytic activities alone might be a substantial limit to the complete understanding of the responses of these enzymes.

Overall, this study has shown the important role of liver and also of extra-hepatic tissues, such as gills and intestine, in the phase I and II metabolism of BaP in Nile tilapia, further supporting their function as barriers for the uptake, metabolism and distribution of xenobiotic compounds.

In order to assess the cooperation between ABC efflux transporters and biotransformation enzymes, we have adopted a genetic approach. Hence, the identification of genes coding for of ABC proteins in Nile tilapia was performed, since these transporters were not yet identified in this species. The presence of the genes encoding for ecotoxicologically relevant ABC efflux transporters (*ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2* and *ABCG2a*) was demonstrated in Nile tilapia (chapter 4). The partial sequences obtained revealed the presence of highly conserved domains, a characteristic found in all vertebrate ABC efflux transporters (Walker-A, C-motif and Walker-B), and phylogenetic analysis showed the clustering with ABC transporter sequences from other fish species. These data allowed the determination of the tissue expression pattern of ABC transporters, *CYP1A* and *GSTα* in Nile tilapia, which showed high levels in tissues that function as pharmacological barriers, such as liver, gills and intestine. Among the ABC efflux transporters studied, *ABCC1*, *ABCC2* and *ABCG2* were ubiquitously distributed throughout the three tissues, although *ABCC1* and *ABCC2* showed slightly higher levels of expression in gill and proximal intestine, respectively. *ABCB1b* and *ABCB11* revealed a more tissue-specific expression pattern. The highest levels of *ABCB1b* expression were recorded in proximal intestine, and no expression was detected in gill. On the contrary, *ABCB11* mRNA expression was almost exclusively confined to the liver, and very low expression was measured in intestine and no expression was detected in gill. The expression pattern of ABC efflux transporters in Nile tilapia tissues follows the ones described for other fish species (Cai et al., 2003; Loncar et al., 2010; Long et al., 2011a; Long et al., 2011b) and mammals (Brady et al., 2002; Gerloff et al., 1998; Keppler and Konig, 1997; Maliepaard et al., 2001; Mottino et al., 2000). High basal levels of steady state *CYP1A* and *GSTα* mRNA were detected in liver and in extra-hepatic tissues, further supporting the fact that these are important locations for xenobiotic metabolism and excretion, as observed in other studies (Kim et al., 2010; Li et al., 2010; Rees et al., 2003; Reynolds et al., 2003), and also supported by the data obtained at the activity level previously described. The mRNA expression pattern of these players of cellular detoxification (ABC transporters and biotransformation enzymes), is in agreement to the xenobiotic absorption and detoxifying abilities described for each organ.

Considering the transcriptional response of ABC efflux transporters to the presence of BaP and/or its metabolites, although *ABCB1b* mRNA has shown a tendency to an increase in liver and intestine of BaP water-exposed animals, up-regulation was not significant, which suggested that, apparently, BaP is not a substrate for Pgp. On the contrary, a clear response to the presence of BaP and/or its metabolites was observed from other ABC efflux transporters, such as *ABCC2* and *ABCG2*. Waterborne exposure to

BaP led to a significant up-regulation of the mRNA transcripts of *ABCC2* in gill and *ABCG2* in liver and intestine. In aquatic organisms, the interaction of environmental contaminants with ABC efflux transporters is still poorly documented, and has been largely confined to the study of Pgp (ABCB1). Despite the limited information on the response of other ABC efflux transporters, some evidence exists on the involvement of these particular transporters in detoxification mechanisms. Hepatic *ABCC2* and *ABCG2* mRNA up-regulation was previously reported in fish inhabiting contaminated sites (Paetzold et al., 2009) and, after *in vivo* exposure to heavy metals an increase of liver *ABCC2* transcripts was also observed in other fish species (Long et al., 2011b; Zucchi et al., 2010). Our findings corroborate these results and go further by showing that ABCC2 and ABCG2 can probably perform similar functions in different barrier tissues. The fact that the majority of BaP metabolites in plasma and bile of water exposed Nile tilapia were phase II conjugates (chapter 3), and also the correlations found between biliary BaP metabolites (chapter 2) and *ABCC2* and *ABCG2* mRNA expressions, points to a role of these transporters in the efflux of phase II metabolites in gill (by ABCC2) and intestine (by ABCG2).

Biotransformation enzymes of phase I and phase II were also evaluated, by means of mRNA expression, in BaP exposed fish. *CYP1A* was significantly up-regulated, in a dose dependent manner, after waterborne exposure to BaP in liver, gills and intestine, and in intestine after dietary exposure. *CYP1A* expression was correlated with CYP1A-associated catalytic activities (chapter 2) in both routes of exposure, thus providing evidence that the mRNA increase was further reflected on the protein functionality. Moreover, *CYP1A* mRNA expression was significantly correlated with *ABCC2* in gill and *ABCG2* in intestine, further supporting the proposed cooperative role of ABC transporters and biotransformation enzymes in these tissues. After waterborne exposure, an increase in *GSTα* mRNA expression was observed in gill, although it was not statistically supported. Moreover, *GSTα* mRNA expression was significantly correlated with *ABCC2* transcripts, in the same tissue, and with biliary BaP metabolites. These data support that ABCC2 efflux of metabolites, in Nile tilapia, may occur preferably through a co-transport with GSH, as described in mammals.

In summary, our results showed that the role of ABCCs and ABCG2 in the efflux of GSH, GA and/or sulphate conjugates, pointed out in mammalian models (Leslie et al., 2005), is apparently maintained in fish species, like Nile tilapia. Moreover, for the first time, indications were shown that ABCC2 may be responsible for the preferential efflux of GSH metabolites in gills. In mammals, ABCG2 has been associated to the efflux of sulphate conjugates, and we must not exclude this possibility in Nile tilapia, since sulphate BaP conjugates were detected in bile (chapter 3) and no correlations were found between this

transporter mRNA expression and *GSTα* mRNA. However more studies are needed to confirm this hypothesis.

To evaluate Pgp (ABCB1) and CYP1A at the protein level, and their cellular localization in Nile tilapia tissues immunochemical methods were applied (chapter 5). Pgp was detected using two different mAb directed to mammalian Pgp, C219 and C494. This kind of approach, using two or more standardized anti-Pgp antibodies that recognize different epitopes, improves the reliability of immunological detection of Pgp (Beck et al., 1996; Lacueva et al., 1998; Valk et al., 1990). These antibodies are known for the different staining patterns, and labelling intensity, usually associated with each Pgp antibody (Ginn, 1996; Van Der Heyden et al., 2009). In face of our results, Pgp cellular localization in Nile tilapia described in this study seems to be in agreement with the majority of the studies conducted in fish and mammals species with both antibodies, which is a positive indication of a specific reaction to Pgp. Despite the tendency to an increase observed in hepatic and intestinal *ABCB1b* mRNA after waterborne exposure to BaP (chapter 4), at protein level, no changes were detected in Pgp expression of exposed animals. Therefore, this can be one more evidence that Pgp is apparently not involved in the efflux of BaP in Nile tilapia tissues, since no significant changes in Pgp protein or *ABCB1b* mRNA expression were observed in liver, nor in intestine of animals exposed to waterborne BaP. In mammals, the interaction of BaP with Pgp is still controversial, some studies indicate that this transporter is involved in the efflux of the parent compound (Chao Yeh et al., 1992; Fardel et al., 1996) while others report the opposite (Buesen et al., 2002; Schuetz et al., 1998). In fish, Zaja et al. (2011) also found no BaP interaction with *ABCB1* in a hepatoma cell line of *Poeciliopsis lucida*, and in catfish liver and intestine, BaP dietary exposure had no effect on Pgp levels (Doi et al., 2001), accordingly to what we have reported in our study. However, since both antibodies may cross-react with other proteins (Childs et al., 1995; Georges et al., 1990; Rao et al., 1994), besides Pgp, results from these studies should be taken with care, as for our study. Findings provided by the mAb C219 do not exclude the possibility of cross-reactivity with Spgp (ABCB11) in Nile tilapia tissues, rather than to Pgp alone, since both proteins share similar molecular weight thus being indistinguishable by western blot. Moreover, we were unable to confirm the size of the protein reacting to the mAb C494, which, consequently, is a drawback on the interpretation of the results provided by this antibody. Nevertheless, considering a positive reaction of C494 antibody to Pgp alone, results showed the possibility of the existence of a different Pgp-isoform in gill, not previously described in other immunochemical studies in fish. mRNA transcripts of Pgp (*ABCB1b*) were not detected in Nile tilapia gill (chapter 4), similarly to other studies in a different fish species (Loncar et al., 2010; Zaja et al., 2008). The existence of more than

one Pgp isoform in fish has been proposed (Dean and Annilo, 2005; Fischer et al., 2011), and could explain the positive reaction observed in gills of Nile tilapia after probing with the mAb C494.

Phase I biotransformation enzyme, CYP1A, was also assessed at protein level by the use of the mAb C10-7. Results showed that CYP1A was localized in liver hepatocytes and vascular endothelium, gills pillar and epithelial cells and intestinal enterocytes and vascular elements of Nile tilapia, which follows the cellular distribution pattern previously described in other fish species (Jönsson et al., 2006; Van Veld et al., 1997). A dose-dependent induction of CYP1A protein levels was observed in liver, gills and intestine after waterborne exposure to BaP, in agreement to the previous results both at gene (chapter 4) and at functional levels (chapter 2). With this approach, we have validated the importance of this phase I enzyme, and also the role of extra-hepatic tissues (gills and intestine) in the first-pass metabolism of waterborne pollutants. ABCB1 and CYP1A were not assessed at protein level in dietary exposed animals, since mRNA expression levels and CYP1A–associated catalytic activity were not altered in the three analysed tissues simultaneously, in opposition to waterborne exposed animals and limited information would be obtained. In summary, at protein level, responses of ABCB1 and CYP1A confirmed the results of the mRNA expression levels and catalytic activities previously measured, and supported that, while BaP is not a substrate for Pgp, CYP1A is involved in its metabolism in the different barrier tissues of Nile tilapia.

The presence and functionality of ABC efflux transporters proteins has been demonstrated in adult stages of other aquatic species (Loncar et al., 2010; Zucchi et al., 2010), but information on the characterization during the early life stages of fish development, which is a particularly sensitive period to the presence of xenobiotic compounds, is still very scarce. Only two studies have reported the sensitivity of ABCC1 and ABCC2 of zebrafish embryos to the presence of heavy metals (Long et al., 2011a,b), but no information exists on the characterization of all cellular detoxification phases during the ontogeny of fish. In order contribute to a more detailed knowledge, we evaluated the mRNA transcripts of ABC transporters and biotransformation enzymes during the development of Nile tilapia embryos and larvae (chapter 6). At these critical stages some of these genes were present since the onset of embryos development (*ABCB1b*, *ABCC1*, *CYP1A* and *GSTα*), thus being of maternal origin, while others were transcribed in later embryonic stages, preceding highly sensitive periods of development (*ABCB11*, *ABCC2* and *ABCG2*). The zygote transcription initiates during the cleavage period which resulted in an increase of mRNA transcription of the maternally transmitted genes during the following stages. At the beginning of the organogenesis (occurring after segmentation

period), *ABCB1b* and *CYP1A* showed a decrease in mRNA expression, probably related to a dilution effect associated with the fact that these genes display a more tissue-specific distribution pattern than the remaining ones, as shown in adult tissues of Nile tilapia (chapter 4), and other fish species (Loncar et al., 2010; Sarasquete and Segner, 2000; Zaja et al., 2008). After 3-4 dpf, during the pharyngula period, the first mRNA transcripts for *ABCB11*, *ABCC2* and *ABCG2* were detected in Nile tilapia. This period precedes the most sensitive stage in fish development, the hatching period, characterized by the mouth opening and the loss of the chorion, implying that embryos become much more exposed to the surrounding environment. The need for an increase of the intrinsic defence mechanisms may be reflected by the increase in the mRNA expression of the majority of the genes evaluated, *ABCB11*, *ABCC1*, *ABCC2*, *ABCG2* and *GSTα*. Higher MXR activity after the hatching period has been previously reported in invertebrate aquatic species (Faria et al., 2011; McFadzen et al., 2000; Minier et al., 2002) and in zebrafish (Long et al., 2011b), which comes in agreement with our results. The presence of genes encoding for ABC efflux transporters since the onset of embryos development indicates that these proteins should play a crucial role in the tolerance against environmental xenobiotics during these critical life stages. Therefore, although more detailed functional studies are needed, these data point to the fact that the presence of toxicants with the ability to block or saturate ABC efflux transporters and/or biotransformation enzymes in early life stages of aquatic organisms, may compromise their normal growth and, therefore, jeopardize their survival.

7.2. Conclusions

Based on the results obtained, the main conclusions driven from this study are:

- The detoxification pathway of pollutants is dependent on the route of exposure, which can have implications in environmental risk assessment studies;
- ABC efflux transporters and biotransformation enzymes act in a cooperation manner for the detoxification of toxic compounds in fish;
- ABC transporters, CYP1A and GST are important defence tools for the survival of living organisms, and are present since the early life stages of development in fish.

These main conclusions were based on the following results:

- Different tissues were involved in the pathway of BaP detoxification in Nile tilapia, depending on whether exposure occurred through the water or through the diet. Waterborne exposure led to phase I metabolism in liver, gills and intestine, while after dietary exposure, intestinal CYP1A metabolism was the major pathway of BaP detoxification. EROD activity and biliary BaP metabolites reflected properly the contamination levels. Liver and extra-hepatic tissues, as gills and intestine display an important role as barriers for the uptake, metabolism and distribution of xenobiotic compounds, thus information provided from studies in these tissues can be highly valuable in environmental risk assessment studies.
- CYP1A was shown to display a very important role in the phase I metabolism of BaP in liver, gills and intestine and phase II metabolism was an effective step on the neutralization of the toxic phase I metabolites into non-toxic forms.
- ABC efflux transporters, *ABCB1b*, *ABCB11*, *ABCC1*, *ABCC2* and *ABCG2a* showed to be present in Nile tilapia tissues involved with xenobiotic absorption, metabolism and excretion. This pattern of expression can reflect the proposed role in the protection against pollutants by regulating their passage and/or metabolites through cellular and tissue barriers.
- While ABCB1 should not be involved in the efflux on BaP and/or its metabolites, ABCC2 and ABCG2a was apparently associated to the efflux of phase II metabolites, probably displaying major roles in gills and proximal intestine of fish, thus reflecting the cooperation between efflux transporters and biotransformation enzymes.
- Genetic information for some ABC efflux transporters and biotransformation enzymes was shown to be maternally transmitted to Nile tilapia embryos, since

mRNA for *ABCB1b*, *ABCC1, CYP1A* and *GSTα* were present since the onset of development, which points to a substantial role of these proteins in protection against pollutants at these critical life stages. Other ABC efflux transporters (*ABCB11, ABCC2* and *ABCG2*) were transcribed in later embryonic periods preceding highly sensitive stages of development, when the demand for these proteins may rise in order to assure the necessary levels of protection against toxicants from the surrounding environment.

7.3. Future perspectives

This work focused on the response patterns of ABC efflux transporters and biotransformation enzymes to the exposure to PAHs in fish. The pattern of response of phase I biotransformation enzyme CYP1A was demonstrated at gene, protein and activity level, therefore creating a very concrete scenario of its role as drug metabolizing enzymes. However, the function of phase II enzymes still requires further studies, in order to properly evaluate which specific isoforms are involved in the detoxification processes.

The study of ABC transporters has been, in part, limited by the inexistence of specific techniques, probes, antibodies that can properly confirm their presence and functionality. Thus, in order to get irrefutable proofs of their way of action, these difficulties must be overcomed by directing research to the development of more specific and appropriate tools for their characterization.

After evaluating the mRNA response of some of the ABC efflux transporters, namely *ABCC2* and *ABCG2*, the hypothesis of them displaying a role in the efflux of phase II metabolites in Nile tilapia has been proposed. Nevertheless, to fully elucidate this response, future functional studies are needed to demonstrate their activity as transporters of BaP metabolites. Moreover, ABCC2 apparently displays a more important role in gills in the efflux of GSH and GA conjugates, while ABCG2 probably preferentially effluxes sulphate conjugates in intestine. Future studies should be directed to the evaluation of phase II enzymes (GSTs, UGTs and SULT), and ABC transporters in order to properly address the proposed affinity of ABCC2 and ABCG2 to the different types of metabolites.

Similarly, although we have shown the temporal expression pattern of ABC efflux transporters and biotransformation enzymes during the developmental stages of Nile tilapia, the role of these proteins should be addressed in future studies. The measurement

of the uptake of fluorescent dyes substrates for ABC transporters by the larvae in the absence/presence of transporter inhibitors, and the evaluation of the associated catalytic activities of drug metabolizing enzymes, are useful tools to confirm the functionality of these proteins in these critical life stages. These procedures can be valuable tools for the identification of chemicals that have the ability to interfere with these detoxification systems, therefore compromising the efficacy of these defence mechanisms. The small size of embrionary forms allows the testing of several different compounds in a reduced size “facility”, reducing the number of adult animals used and the volume of wastes produced in this type of studies.

Despite ABCB1 is the most studied ABC efflux transporter, and previous works reported a role in the efflux of PAHs, our findings seem to point to the fact that BaP should not be a substrate for Pgp. Nevertheless, results at protein level for Pgp were not completely enlightening. In this field, the inexistence specifically-designed probes for ABC efflux transporters acts as a limiting factor to clearly demonstrate their cellular localization and response patterns at the protein level. Therefore, there is the need to further studies directed to unravel their response at protein level by the use of fish-specific antibodies directed to ABCB1, ABCCs and ABCG2.

The possible cooperation between ABC efflux transporters and biotransformation enzymes of phase I and phase II in the detoxification pathway of PAHs in fish has been addressed in this study, and demonstrated at transcriptional level by the response patterns of ecotoxicologically relevant ABC transporters. Future studies directed to the evaluation of the transcriptional regulation of ABC efflux transporters and biotransformation enzymes, may shed some light on the possible cooperation and coordination between these groups of proteins, with paramount roles in cellular detoxification.

7.4. References

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Appendix

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Comparision of the waterborne and dietary routes of exposure on the effects of Benzo(a)pyrene on biotransformation pathways in Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

BaP is one of the most studied PAH, due to its ubiquitous presence in aquatic environments and toxicity to aquatic organisms. The main goal of this study was to assess BaP effects in Nile Tilapia after waterborne and dietary exposures, through the evaluation of EROD and GST activities in liver, gills and intestine, and BaP metabolites in bile; and also to evaluate the usefulness of these commonly used biomarkers after two different routes of exposure. Waterborne exposure to BaP led to a significant induction of EROD in all tissues analyzed (644%, 1640% and 2880% in relation to solvent in liver, gill and intestine respectively) while in dietary exposures EROD was induced only in intestine (3143%) after exposure to high BaP concentrations. GST activities with CDNB were slightly induced in liver (40%) and in gill (66%) after water exposure to BaP, and in intestine after dietary exposure to low BaP concentrations (182%). BaP metabolites in bile increased after both exposure routes, and were highly correlated with EROD activity after water exposure. In summary, this work has shown that the effects of BaP on biotransformation pathways depend on the route of exposure. Moreover, barrier tissues like gills and intestine also have an important role in the first-pass metabolism of BaP, reducing the amount of parent compound that reaches the liver to be metabolized. For that reason, EROD activity as a biomarker of exposure should also be applied in extrahepatic organs, like gills and intestine, in monitoring studies. Biliary BaP type metabolites are good reflectors of contamination levels under both exposure routes, while GST activity with CDNB as substrate, as a phase II enzyme, does not seem a reliable biomarker of exposure to BaP regardless the route of exposure.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are prevalent environmental contaminants commonly found in aquatic sediments and waters associated with urbanized estuarine and coastal areas (Meador et al., 1995). Although these contaminants can arise from natural sources, such as oil seeps, volcanoes and forest fires, anthropogenic sources as vehicle exhaust, power generation and oil pollution are the main causes for environmental PAH contamination (Latimer and Zheng, 2003). In aquatic organisms, the uptake of pollutants can occur through the contact of contaminated food, water and sediments (Bruggeman et al., 1984; Hendricks et al., 1985), and the degree of toxicity can be influenced by the route, by the dose and by the duration of exposure (Grimmer et al., 1988; Driver et al., 1991; Bloomquist, 1992). One of the most common and toxic PAH in the aquatic environments is Benzo(a)pyrene (BaP) and its carcinogenic and mutagenic properties are widely studied (Buhler and Williams, 1989; Tsukatani et al., 2003). The great majority of the studies focusing on BaP effects in the detoxification mechanisms in aquatic organisms have used intraperitoneal injections (Van Der Weiden et al., 1994; Pacheco and Santos, 1998; Nacci et al., 2002; Wang et al., 2006; Wang et al., 2008; Nahrgang et al., 2009) as the route of exposure. Some studies have investigated the effects on the detoxification mechanisms of BaP after dietary (Hendricks et al., 1985; Wolkers et al., 1996; Reynolds et al., 2003), and waterborne exposure routes (Levine and Oris, 1999; Wu et al., 2007; Ortiz-Delgado et al., 2008; Wang et al., 2008) which are two of the most important routes of pollutants uptake in aquatic organisms. However, to our knowledge, there is still a lack of studies that directly compare the biochemical effects

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of different concentrations of the same contaminant after both exposures routes in the same species.

In fish, as in other vertebrates, exposure to PAHs results in the induction of enzymatic systems involved in the metabolism of the xenobiotic compound, for the detoxification of the organism (Black and Coon, 1987; Buhler and Williams, 1989) due to a high-affinity of these pollutants to bind the aryl-hydrocarbon receptor (AhR). Studies in mammals have shown that the activation of the AhR-dependent signal transduction pathway controls the expression of several genes, including cytochrome P4501A (CYP1A) and some glutathione-*S*-transferase (GST) genes (George, 1994; Hankinson, 1995; Schmidt and Bradfield, 1996). Like in mammals, in fish these genes are believed to be primarily involved in hepatic biotransformation of pollutants, like PAHs (reviewed in van der Oost et al., 2003). The measurement of phase I and phase II biotransformation enzymes hepatic activities, have been widely applied as biomarkers of biochemical effects, and as a complement to chemical analysis (Richardson et al., 2001; Ferreira et al., 2006, 2008; Jönsson et al., 2009; Nahrgang et al., 2009; Reis-Henriques et al., 2009). However, despite the fact that the liver is considered to be the main site of CYP1A expression, its expression and induction has been observed in other tissues as well, including those in direct contact to the environment, such as gills (Jönsson et al., 2004, 2006; Nahrgang et al., 2010a,b) and intestine (McElroy and Kleinow, 1992; James et al., 1997). Nevertheless, the relative importance of branchial and intestinal uptake route to the overall exposure remains poorly understood. After PAH metabolism in the liver, their metabolites are secreted into the bile, stored in the gall bladder and excreted to the intestinal tract (Au et al., 1999). Therefore, due to their high metabolism rate in fish (Meador et al., 1995), PAHs tend not to bioaccumulate in tissues, and the direct quantification of these compounds in fish tissues may not yield an accurate estimation of contaminant exposure and uptake. As a result, the measurement of PAHs metabolites in fish bile is considered a reliable method for assessing the degree of exposure of the organisms to these type of pollutants (Krahn et al., 1986). In this study, Nile tilapia (*Oreochromis niloticus*) was used as model species. Besides being an economically important cultured species, namely in Asia and Africa, Nile tilapia is also a well established model in many toxicological studies (Almeida et al., 2001; Straus, 2003; Coimbra et al., 2005, 2007; Figueiredo-Fernandes et al., 2006).

The main objectives of the present work were to evaluate differences in the detoxification mechanisms in juvenile Nile tilapia, after waterborne and dietary exposure routes to BaP. Biochemical effects of BaP were assessed, by means of 7-ethoxyresorufin *O*-deethylase (EROD) and glutathione-*S*-transferase (GST) activities as a measure of phases I and II of the biotransformation mechanism, in liver, gills and intestine. Additionally, fixed wavelength fluorescence (FF) was used to quantify BaP type metabolites as fluorescent aromatic compounds (FACs) in bile.

2. Materials and methods

2.1. Chemicals

Benzo(a)pyrene (99% purity), resorufin sodium salt, 7-ethoxyresorufin, ß-NADPH (95% purity), BSA (99% purity), 1-chloro-2,4-dinitrobenzene (CDNB, 97% purity), α-Dithiothreitol (99% purity), GSH (99% purity) were purchased from Sigma Aldrich, Germany. All the other chemicals were of analytical grade, and were purchased from local companies.

2.2. Animals

All animals used in this study were born and raised in the laboratory (CIIMAR, Porto, Portugal). The corresponding breeders stock was obtained from the Aquaculture Station of UTAD (Universidade de Trás-os-Montes, Portugal). Fish used in all assays were juveniles, and therefore not sexually mature, as confirmed by macroscopic analysis of the gonads when animals were sacrificed. Until the start of the exposure assays, fish were kept in 60 L aquaria supplied with biological filtration. Prior to the experiments, animals were randomly distributed in the experimental aquaria (30 L), and submitted to an acclimatation period of one week. All tanks were supplied with continuous aeration to maintain dissolved oxygen near saturation. Dechlorinated tap water was used at a temperature of 20 ± 2 °C, with a 12 h:12 h (light:dark) photoperiod, and fish were fed commercial food pellets (Aquasoja, Portugal) until satiation, once a day.

2.3. Stock solutions of BaP and preparation of contaminated food

For the waterborne exposures, stock solutions of BaP were prepared in acetone (0.5, 1.25, 2.5 and 5 g L^{-1}) and were administered directly in the experimental aquaria. The percentage of solvent added in the experimental aquariums was 0.002%. For the dietary exposures stock solutions of BaP with concentrations of 0.1 and 40 g L^{-1} were prepared, respectively for the first and second assays. The contaminated diets were prepared by immersion of food pellets in BaP stock solutions diluted in acetone, in a proportion of 0.32 mL g^{-1} of food. For control groups, nothing was added to the food pellets, and for solvent control groups only acetone was added to the food pellets. Acetone was evaporated under air current for 24 h, until the pellets were completely dry, and diets were stored at −20 °C until further use.

2.4. Xenobiotic exposures

For the water exposure, juvenile Nile tilapia (N = 116, average weight of 14.2 ± 0.6 g, average length of 9.30 ± 0.14 cm) were exposed to nominal water concentrations of 10, 25, 50 or 100 μg of BaP/L for 14 d. Also, a control group and a solvent control group (only solvent was added) were maintained. All treatments were performed in duplicate, with two exposure tanks per concentration. Waterborne exposures were conducted in semi-static conditions in 30 L aquaria. Daily, 80% of the water was renewed, followed by the addition of fresh BaP solution or solvent to each one of the treatment groups. Animals were fed to satiation every 2 d, with the exception of the day before sampling. Sampling was performed prior to the first BaP addition, and 7 and 14 d after the contaminant addition.

For dietary exposures two experiments were conducted, and fish were maintained in 30 L aquaria in continuous water flow conditions, which assured 100% of water renewal per day. In the first assay, juvenile tilapia (N = 72, average weight of 11.21 ± 1.00 g, average length of 8.47 ± 0.27 cm) were exposed to 1 and 10 μg of BaP g^{-1} of food for 14 d. In the second dietary assay, fish (N = 129; average weight: 11.15 ± 0.42 g, average length of 8.49 ± 0.09 cm) were exposed to 100 and 200 μg of BaP g^{-1} of food for 21 d. Fish were sampled before the addition of BaP contaminated diets, and at days 7, 14 and 21 (only in the second experiment). In both assays a control (uncontaminated food) and a solvent control group (acetone alone) were maintained, and duplicates were made for each treatment, with two exposure tanks per concentration. Fish were fed daily at a rate of 3% of body weight. After feeding, animals were observed, to assure that the total of the food was consumed in 2 to 3 min. After every sampling point, animals were weighted in order to recalculate the amount of food necessary for each tank, to maintain the percentage of feeding at 3% body weight.

2.5. Sampling

Fish were anesthetized on ice cold water and sacrificed by decapitation, and body weight and length were recorded for subsequent calculation of condition factor (CF = body weight (g) × 100/body length^3 (cm)). Liver, gills and intestine were excised from the animal, and bile was collected from the gall bladder with a 1 mL syringe. Liver weight was recorded for the calculation of hepatic somatic index (HSI = liver weight (g)/body weight (g) ×100). Liver, gills, intestine, and bile were immediately frozen in liquid nitrogen, and stored at −80 °C until further use. CF and HSI prior to the beginning of the water assay were 1.66 ± 0.04 and 1.33 ± 0.11, respectively. Before the dietary exposures, CF and HSI were 1.62 ± 0.07 and 1.21 ± 0.07 for the first assay, and 1.68 ± 0.03 and 1.95 ± 0.05 for the second assay, respectively. No significant differences were seen in these parameters during the course of the experiments, indicating that the overall condition of the animals was maintained during the experimental periods (Ricker, 1975; Grant and Brown, 1999).

2.6. BaP determination in water and food samples

To determine the real concentration of BaP in the water of the experimental aquaria, BaP was extracted from water samples according to the method described by Cheikyula et al. (2008), and assessed by the method of Rey-Salgueiro et al. (2008). After the addition of the contaminant, the real concentrations of BaP in water samples were 10.49, 22.51, 41.46 and 84.77 μg L^{-1}, respectively for the nominal concentrations of 10, 25, 50 and 100 μg L^{-1}. After 24 h, and before the addition of fresh contaminant to the aquaria, the amount of BaP still present in each one of the treatment groups was 0.34, 0.69, 12.19 and 25.70 μg L^{-1}. In water collected from control and solvent control groups no BaP was detected at both times.

Food pellets supplied to the fish in dietary exposures were also analyzed in terms of BaP concentration, according to the method described by Rey-Salgueiro et al. (2009). The real BaP concentrations in the food pellets were 0.06, 0.07, 0.42, 3.99, 36.24 and 92.70 μg g^{-1}, respectively for control, solvent, 1, 10, 100 and 200 μg g^{-1}.

2.7. Biochemical analysis

EROD activity was measured according to Ferreira et al. (2008) in the microsomal fraction of liver, gills and the first one-third of the intestine. Briefly, tissues were homogenized in ice cold buffer (50 mM Tris–HCl, 0.15 M KCl, pH 7.4), and microsomes were obtained by centrifugation of the 9000*g* supernatant at 36 000*g* for 90 min at 4 °C. The pellet was then ressuspended in buffer (50 mM Tris–HCl, 1 mM Na_2EDTA, 1 mM dithiothreitol, 20% v/v glycerol pH 7.4) and spun down at 36 000*g* for 120 min (Bucheli and Fent, 1995). Microsomes were suspended in EDTA-free resuspension buffer and stored at −80 °C until use. Microsomal suspension (20 μL), with average protein concentration of 3.01 ± 0.20 mg mL^{-1}, was incubated with ethoxyresorufin (2 μM in 50 mM Tris–HCl, 1 mM dithiothreitol, 0.15 M KCl, pH 8.0) for 1 min, and the enzymatic reaction was initiated by the addition of NADPH 4.5 μM. EROD activity was measured for 5 min at λ_{ex}530 nm and λ_{em}585 nm, by fluorometry, and determined by comparison to a resorufin standard curve (concentrations range from 3 to 100 nM). Hepatic EROD activity was expressed in pmol min^{-1} mg^{-1} protein. During the procedure of microsomes isolation, an aliquot of the cytosolic fraction (obtained after the 9000*g* centrifugation) was separated for the analysis of GST activity. GST was determined according to the method of Habig et al. (1974), adapted to microplates as described in Ferreira et al. (2008), using glutathione (GSH) 10 mM in phosphate buffer 0.1 M, pH 6.5, CDNB 60 mM in ethanol prepared just before the assay. The reaction mixture was composed by 1.5 mM GSH and 1.5 mM CDNB in phosphate buffer. In the microplate, 0.2 mL of the reaction mixture was added to 0.1 mL of sample, corresponding to 0.3 mg of protein, with a final concentration of 1 mM GSH and 1 mM CDNB in the assay. The GST activity was measured immediately every 20 s, at 340 nm, during the first 5 min, and calculated in the period of linear change of the absorbance. GST activity was expressed in nmol min^{-1} mg^{-1} protein. In each treatment group, EROD and GST activities were analysed in 4–10 individuals, depending on the tissue. Protein content in all assays was determined by Lowry method (Lowry et al., 1951), in the correspondent fraction.

2.8. BaP metabolites in fish bile

BaP metabolites in the bile were determined through fixed wavelength fluorescence (FF). Bile samples from control and solvent control of water exposed animals, and from food exposed groups were diluted 1:1000 in ethanol 48%. Bile from water exposed animals was further diluted to 1:10000 (10 and 25 μg L^{-1}) and 1:100000 (50 and 100 μg L^{-1}). FF was performed at the excitation/emission wavelength pair 380/430 nm, since BaP metabolites are more efficiently detected at these wavelengths (Krahn et al., 1993; Lin et al., 1996). Measurements were performed on a BIOTEK SFM25 fluorimeter. The FF values were expressed as arbitrary fluorescence units (a.f.u.) after subtracting the signal levels of the solvent. The bile pigment biliverdin was measured at 380 nm in all samples to estimate bile density, and BaP metabolites fluorescence was normalised to biliverdin, to check if differences in fluorescence intensity could result from differences in bile densities. BaP metabolites were analysed in bile of 4–10 individuals of each treatment group.

2.9. Statistical analysis

Exposure time and treatment effects were evaluated by means of a one-way ANOVA for each one of the mentioned factors, followed by a multiple comparison test (Tukey's test) at a 5% significance level. Some data had to be log transformed in order to fit ANOVA assumptions. Correlations and all the tests were performed using the software Statistica 7 (Statsoft, Inc., 2004). No differences were observed between replicates of the same treatment, nor in control treatments between days, nor between control and solvent control during the exposure time. Therefore, in all biomarkers analysed, results were presented as percentage in relation to solvent control only. Results were shown as mean ± standard error.

2.10. Ethics statement

The animals used in the research that is described in this paper were treated in accordance with the Portuguese Animals and Welfare Law (Decreto-Lei n° 197/96) approved by the Portuguese Parliament in 1996.

3. Results

Mean control values of EROD activity in all assays were 38.09 ± 3.85, 2.09 ± 0.23 and 1.10 ± 0.10 pmol min^{-1} mg^{-1} of protein, respectively for liver, gill and intestine. GST had mean activities in control groups of 120.79 ± 4.46, 63.96 ± 3.51 and 49.16 ± 3.73 nmol min^{-1} mg^{-1} of protein, in liver, gill and intestine, respectively. BaP metabolites in bile were 4584 ± 410 a.f.u. in control animals.

3.1. Water exposure

Biomarkers evaluated after waterborne exposure to BaP are shown in Fig. 1 (EROD activity), 2 (GST activity) and 3a (BaP metabolites in bile).

Results show that water exposure caused BaP dose-related increases of EROD activity in liver (Fig. 1a), gills (Fig. 1b) and intestine (Fig. 1c). Moreover, EROD activity was, in most of the treatments, significantly different from solvent control ($p < 0.05$) in all three tissues. Exceptions were exposure to 10 and 25 μg L^{-1} at day 14 in liver, and in intestine exposure to 10 μg L^{-1} (at days 7 and 14) and 25 μg L^{-1} (at day 7). Regarding the effects of time of exposure, a significant increase between days 7 and 14 was seen in gills and intestine after exposure to 25 μg L^{-1} of BaP. When comparing the extent of EROD activity increase over the solvent control, maximum increases were seen in intestine (from 105% to 2876% increase, Fig. 1c), followed by gills (from 544% to 1639% increase, Fig. 1b) and liver (from 70% to 644% increase, Fig. 1a).

Regarding phase II enzyme, GST, its activity, increased significantly ($p < 0.05$) in liver (after 14 d of exposure – Fig. 2a) and gills (after 7 d of exposure – Fig. 2b) upon exposure to 100 μg L^{-1} of BaP (39 and 66% increases respectively). At day 7 no differences were seen in GST intestine activity, but after 14 of exposure its activity decreased significantly ($p < 0.05$) after exposure to 50 and 100 μg L^{-1}, achieving a maximum decrease over solvent control of 64% after exposure to 100 μg L^{-1} of BaP (Fig. 2c).

BaP metabolites in bile of exposed animals (10, 25, 50 and 100 μg L^{-1}) were always significantly higher than the levels recorded in solvent, and increased with the concentration of the contaminant and with the time of exposure, with the exception of

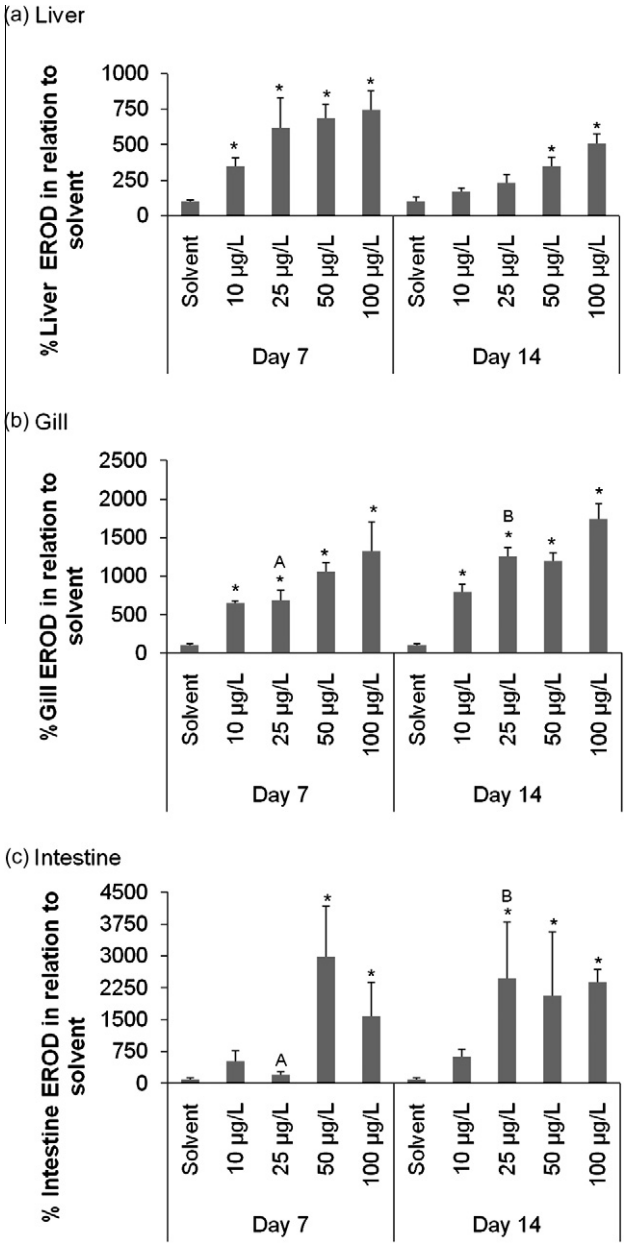

Fig. 1. EROD activities in liver (a), gill (b) and intestine (c) of Nile Tilapia after waterborne exposure to BaP (Solvent, 10, 25, 50 and 100 **μg L^{-1}**). Values are expressed in percent of activity (**pmol min^{-1} mg^{-1}** of protein) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (*) denote significant differences ($p < 0.05$) in comparison to solvent. Capital letters denote significant differences ($p < 0.05$) between days within the same treatment. $n = 4–10$.

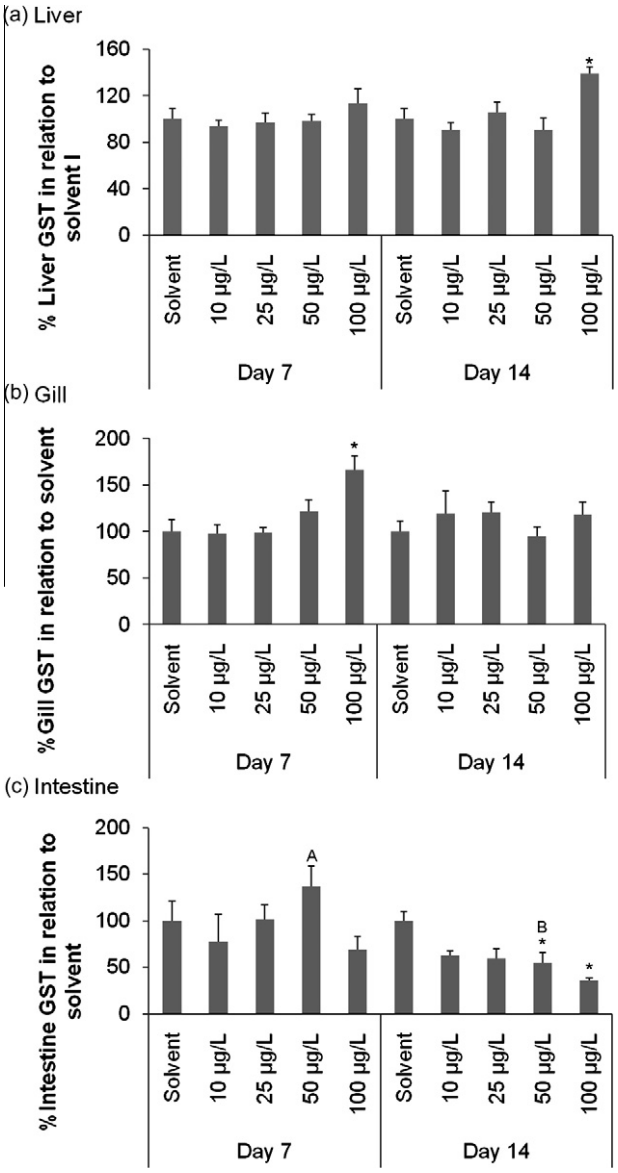

Fig. 2. GST activities in liver (a), gill (b) and intestine (c) of Nile Tilapia after waterborne exposure to BaP (Solvent, 10, 25, 50 and 100 **μg L^{-1}**). Values are expressed in percent of activity (nmol **min^{-1} mg^{-1}** of protein) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (*) denote significant differences ($p < 0.05$) in comparison to solvent. Capital letters denote significant differences ($p < 0.05$) between days within the same treatment. $n = 4–10$.

100 μg L^{-1} at day 14 (Fig. 3a). Results showed that after 7 d of exposure to 10 μg L^{-1}, BaP type metabolites were 83 times higher when comparing to the solvent (8256% increase), and the maximum levels were seen after 14 d of exposure to 50 μg L^{-1}, with a 356 fold increase (35 590%). Moreover, BaP metabolites showed significant positive correlations ($p < 0.05$) with EROD activity in liver ($r = 0.92$), gills ($r = 0.89$) and intestine ($r = 0.65$).

3.2. Diet exposure

Results of EROD and GST activities in Nile tilapia after dietary exposure to 1 and 10 μg of BaP g^{-1} of food are shown in Figs. 4 and 5, respectively.

EROD activity in exposed animals was not different from solvent control during the exposure periods in any of the tissues analyzed (Fig. 4). A significant decrease in EROD activity was seen from day 7 to day 14 at 1 μg g^{-1} of BaP in liver (Fig. 4a) and in gills (Fig. 4b). Phase II enzyme activity, GST, did not change in liver (Fig. 5a) nor in gill (Fig. 5b), and was only induced in the intestine, and after 7 d of exposure to 10 μg g^{-1}; however this induction was not sustained until day 14 (Fig. 5c). Regarding BaP metabolites in bile (Fig. 3b), BaP exposed groups showed higher levels when comparing to the solvent group, with significant differences at 10 μg g^{-1} of BaP at days 7 (149% increase) and 14 (158% increase) ($p < 0.05$).

EROD and GST activities in liver, gills and intestine measured after exposure to 100 and 200 μg of BaP g^{-1} of food are displayed in Figs. 6 and 7, respectively.

In liver (Fig. 6a), results point to a pattern of EROD activity reduction after dietary exposure to 100 and 200 μg of BaP g^{-1} of food. This reduction was statistically supported at day 7 after

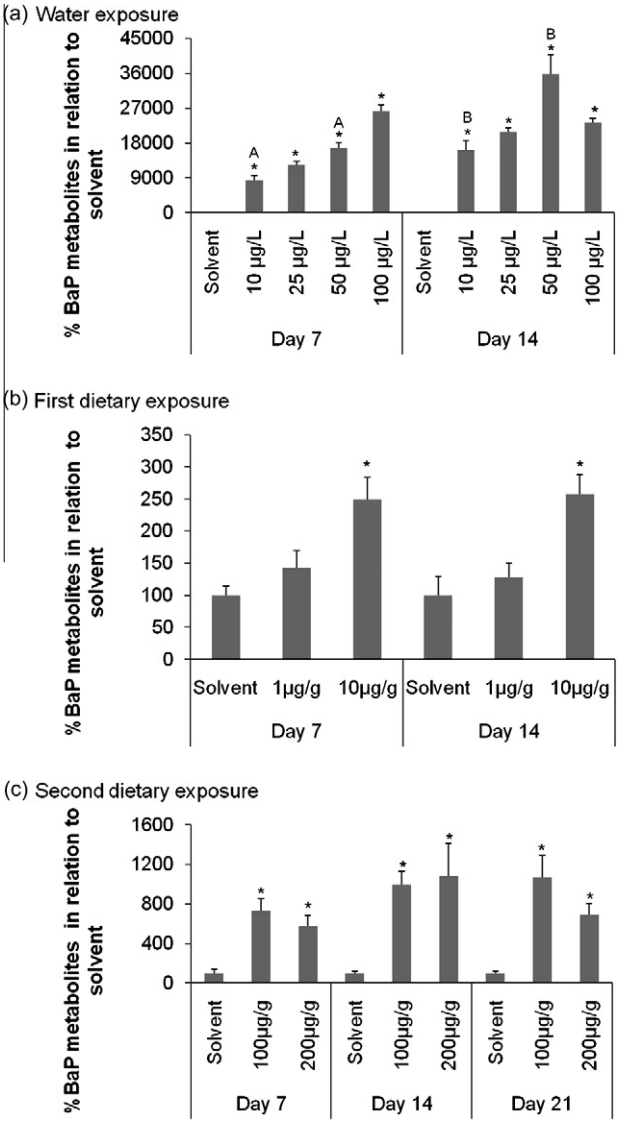

Fig. 3. BaP type metabolites in bile after waterborne exposure to BaP (a), first dietary exposure to BaP (b) and second dietary exposure to BaP (c). Values are expressed percent of arbitrary units of fluorescence (a.f.u.) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (*) denote significant differences ($p < 0.05$) in comparison to solvent. Capital letters denote significant differences ($p < 0.05$) between days within the same treatment. $n = 4–10$.

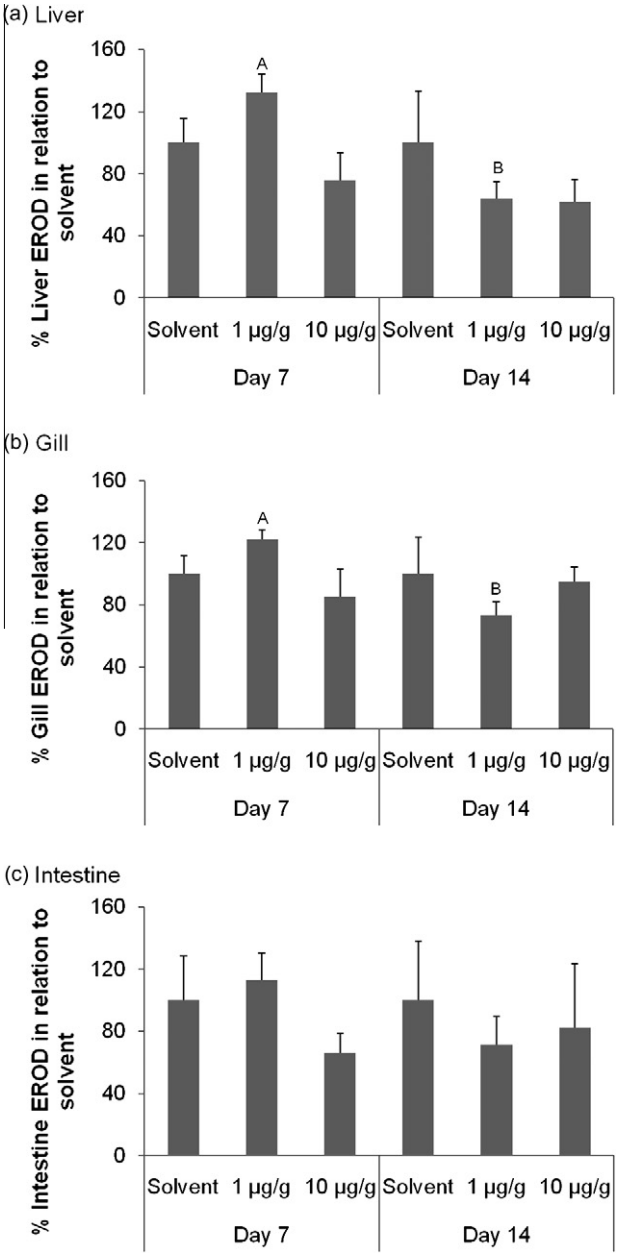

Fig. 4. EROD activities in liver (a), gill (b) and intestine (c) of Nile tilapia after the first dietary exposure to BaP (Solvent, 1 and 10 μg of BaP **g^{-1}** of food). Values are expressed in percent of activity (**pmol min^{-1} mg^{-1}** of protein) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (*) denote significant differences ($p < 0.05$) in comparison to solvent. Capital letters denote significant differences ($p < 0.05$) between days within the same treatment. $n = 4–8$.

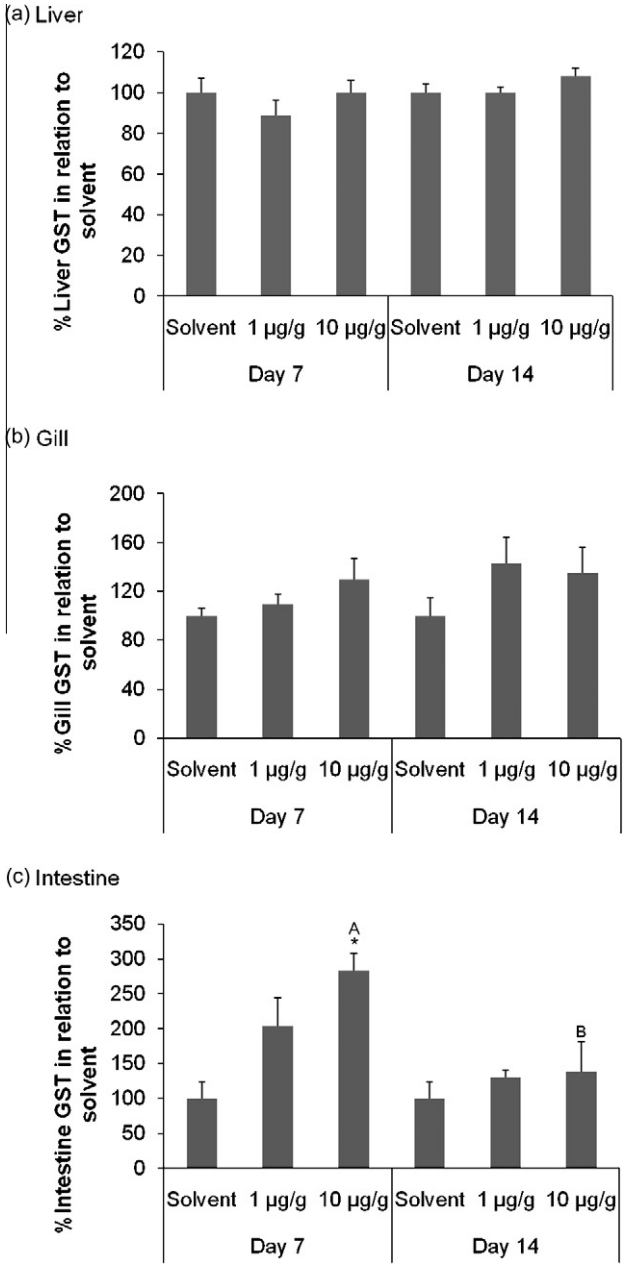

Fig. 5. GST activities in liver (a), gill (b) and intestine (c) of Nile tilapia after the first dietary exposure to BaP (Solvent, 1 and 10 µg of BaP $\mathbf{g^{-1}}$ of food). Values are expressed in percent of activity (nmol $\mathbf{min^{-1}\ mg^{-1}}$ of protein) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (∗) denote significant differences ($p < 0.05$) in comparison to solvent. Capital letters denote significant differences ($p < 0.05$) between days within the same treatment. $n = 4–8$.

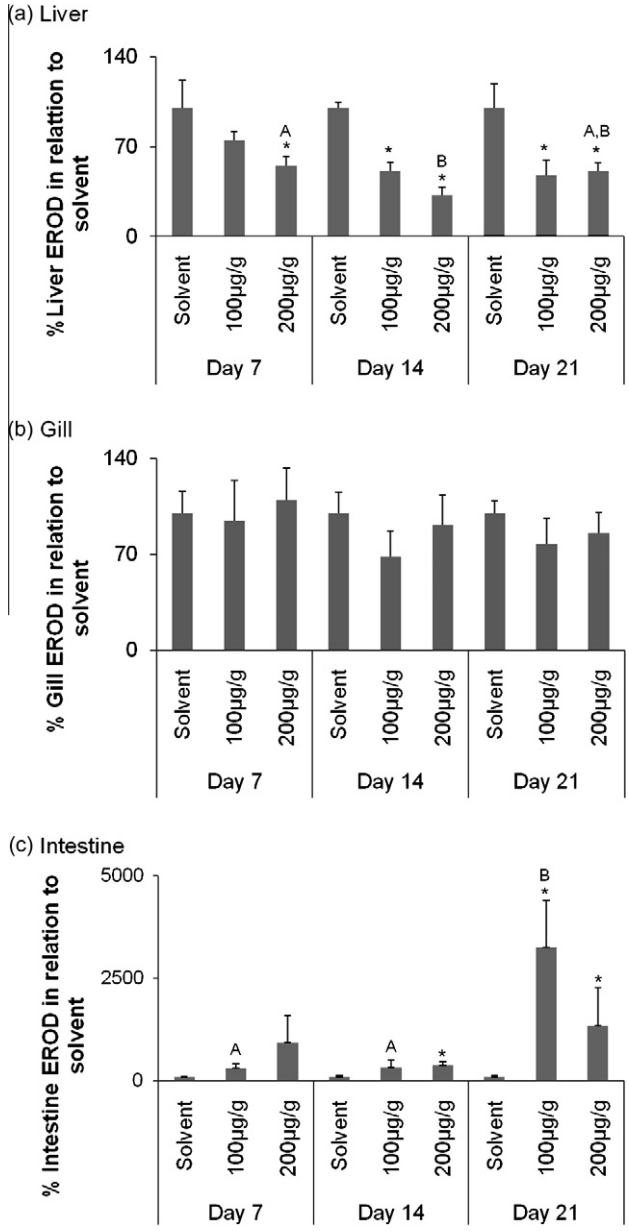

Fig. 6. EROD activities in liver (a), gill (b) and intestine (c) of Nile tilapia after the second dietary exposure to BaP (Solvent, 100 and 200 µg of BaP $\mathbf{g^{-1}}$ of food). Values are expressed in percent of activity ($\mathbf{pmol\ min^{-1}\ mg^{-1}}$ of protein) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (∗) denote significant differences ($p < 0.05$) in comparison to solvent. Capital letters denote significant differences ($p < 0.05$) between days within the same treatment. $n = 4–10$.

exposure to 200 µg of BaP g^{-1} (of food 45% decrease), and at days 14 and 21 to both BaP concentrations ($p < 0.05$), with a maximum decrease of 68% after 14 d of exposure to 200 µg g^{-1}. Moreover, lower levels of EROD activity were detected in 200 µg g^{-1} than in 100 µg g^{-1} at days 7 and 14, and at day 21 similar levels of EROD were recorded in both concentrations. Gill EROD activity did not change during the exposure to dietary BaP (Fig. 6b). However, a significant increase of intestinal EROD activity was seen in exposed animals during the course of the experiment, with a maximum increase occurring after 21 d of exposure to 100 µg g^{-1} (3142% increase over solvent control). Furthermore, there was a significant increase in intestinal EROD activity from day 14 to day 21 at this BaP concentration (Fig. 6c).

High dietary exposures caused no significant changes in GST activity in any of the tissues analysed, as it can be seen in Fig. 7. BaP type metabolites (Fig. 3c) were always significantly higher in exposed animals (100 and 200 µg g^{-1}), with increases in relation to solvent ranging from 471% (200 µg g^{-1}, day 7) to 980% (200 µg g^{-1}, day 14).

4. Discussion

In recent years, there has been an increasing concern over the possible degradation of aquatic environments due to chemical pollution caused by PAHs, among other pollutants. The main reasons for that concern lie in the fact that these are widely spread contaminants with a high toxic potential for living organisms (Icarus Allen

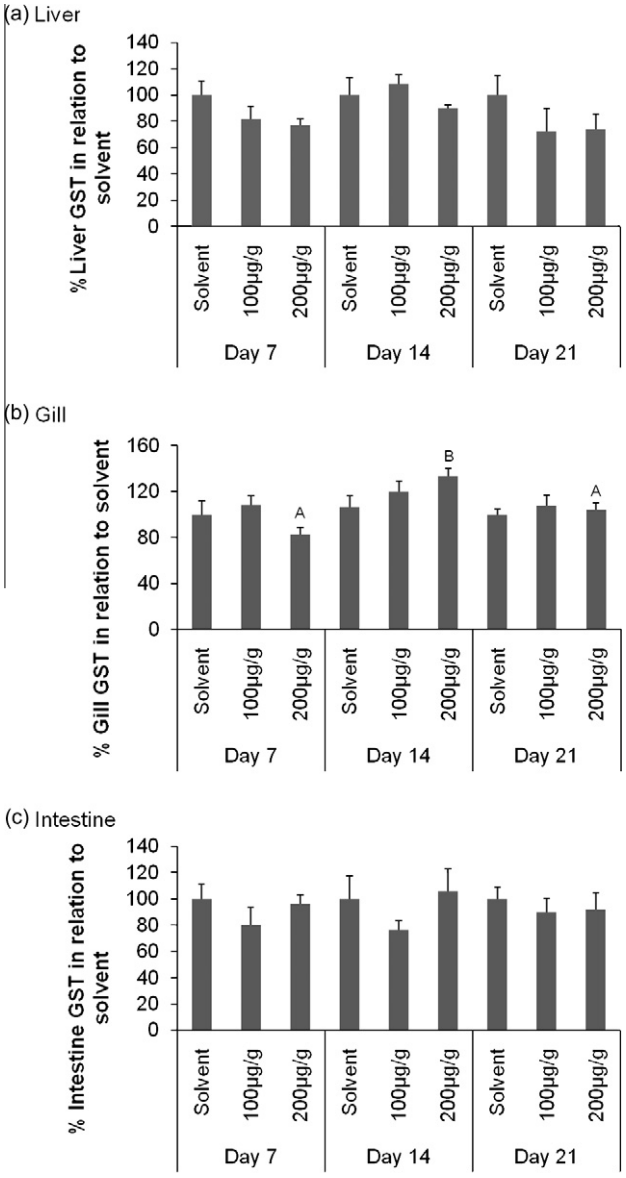

Fig. 7. GST activities in liver (a), gill (b) and intestine (c) of Nile tilapia after the second dietary exposure to BaP (Solvent, 100 and 200 μg of BaP $\mathbf{g^{-1}}$ of food). Values are expressed in percent of activity (nmol $\mathbf{min^{-1}}$ $\mathbf{mg^{-1}}$ of protein) in relation to solvent set to 100%, and are shown as mean ± standard error. Asterisks (∗) denote significant differences ($p < 0.05$) in comparison to solvent. Capital letters denote significant differences ($p < 0.05$) between days within the same treatment. $n = 4–9$.

and Moore, 2004). As a result, increased efforts have been made in order to understand the causal relationships between contaminant exposure and measurable biological and biochemical effects in aquatic organisms, with the integrated use of biomarkers of exposure. In the present study we aimed to achieve a deeper understanding of the effects of BaP in Nile tilapia, after two routes of exposure, waterborne and dietary exposures, and also to assess the usefulness of widely used biomarkers in monitoring studies.

After water exposure to BaP, EROD activity in liver (Fig. 1a), gills (Fig. 1b) and intestine (Fig. 1c) increased during the exposure period. These results show that waterborne exposure led to BaP metabolism in liver and also in extrahepatic tissues, as gills and intestine. Liver is considered to be the main metabolizing organ in fish, having larger amounts of CYP1A enzymes than other tissues (as gills and intestine) (Hinton et al., 2008), and induction of hepatic EROD activity upon exposure to PAHs has been described in other studies (Whyte et al., 2000; van der Oost et al., 2003; Ortiz-Delgado et al., 2008; Kopecka-Pilarczyk and Correia, 2009). This is in agreement with our study since hepatic EROD was strongly induced after exposure to waterborne BaP, and the levels of EROD activity were considerably higher in liver than in gill or intestine. The strong gill EROD induction observed after waterborne exposure to BaP, also indicates that a rapid absorption of this compound occurs in this tissue, and supports the idea that gills work in the first-pass metabolism of BaP. Similar results, with gill EROD induction following BaP exposure, were obtained in other fish species as polar cod (Nahrgang et al., 2010a) and rainbow trout (Jönsson et al., 2006). EROD induction observed in the intestine of BaP exposed animals was due to ingested contaminated water, that was metabolized by intestine CYP1A enzymes, before reaching the liver. Although Nile tilapia is a freshwater specie, and therefore a specie who does not drink water in large quantities, there is always some ingestion of water during feeding and breathing behaviours. Furthermore, our results are in agreement with immunohistochemical studies that compared the cellular expression of CYP1A in different tissues, and that showed that exposure to waterborne BaP resulted in high staining in gill pillar cells and hepatocytes and mildly staining in gut mucosal epithelium (Van Veld et al., 1997).

Some authors have shown that gill EROD activity turned out to be more sensitive than the liver in terms of CYP1A induction to waterborne BaP (Jönsson et al., 2006). The same happened in our study, with gill and intestine showing to be more sensitive than liver to the presence of BaP, which strengthens the idea that it becomes useful to measure EROD activity in extrahepatic tissues, that are involved in the first-pass metabolism of this compound (Levine and Oris, 1999).

After dietary exposures to BaP, the most significant result was the induction of EROD activity in the intestine, after exposure to the highest BaP concentrations (Fig. 6c). This type of response, with induction of intestinal but not hepatic EROD activity, have also been shown in other studies in liver (Van Veld et al., 1987; Reynolds et al., 2003) and intestine (Van Veld et al., 1987; McElroy and Kleinow, 1992; James et al., 1997) of fish exposed to dietary PAHs. Also, immunohistochemical studies showed that exposure to dietary BaP resulted in moderate CYP1A staining in liver but high intensity staining in gut mucosal epithelium (Van Veld et al., 1997; Ortiz-Delgado et al., 2005). We believe that, in our study, the absence of hepatic EROD induction following dietary exposures maybe directly related to partial BaP biotransformation in the intestine (by CYP1A enzymes), limiting the amount of parent compound that reaches the liver for hepatic metabolism, as confirmed by the induced intestinal EROD activity of these animals. However, in the second dietary assay, a reduction of hepatic EROD activity was seen after exposure to high BaP concentrations (Fig. 6a), being statiscally significant at both concentrations at day 21. This situation could have been a result of protein degradation caused by the high concentrations of BaP used (reviewed in Whyte et al., 2000), or due to suppression of CYP1A at a post-transcriptional level mediated through a down regulation of the CYP1A protein (Schlezinger and Stegeman, 2001). On the other hand, it is well known that BaP biotransformation can originate more toxic metabolites (Bauer et al., 1995; Kim et al., 1998; Morthy et al., 2003), and it is possible that part of this metabolites (formed mostly after intestinal CYP1A metabolism) could have entered systemic circulation (Kleinow et al., 1998), causing toxic effects at hepatic level that may have reflected in the lower EROD activities of this tissue. However, although the decrease of hepatic EROD activity was statistically significant, it was relatively weak, leading us to also consider the hypothesis of not being physiologically relevant.

BaP type metabolites measured in bile, increased after water exposure to BaP (Fig. 3a), and were highly correlated with liver ($r = 0.92$; $p < 0.05$), gill ($r = 0.89$; $p < 0.05$) and intestine ($r = 0.65$;

$p < 0.05$) EROD activities. The liver correlation corroborates the fact that exposure of fish to waterborne BaP, leads to a pathway of high hepatic metabolism by CYP1A enzymes. Moreover, correlations between EROD and bile metabolites in extrahepatic tissues also suggest that the majority of BaP metabolites formed in gill and intestine are reabsorbed into the blood stream and then released in the gall bladder. Regarding BaP type metabolites in the dietary experiments, an increase was also seen in BaP exposed animals (Fig. 3b and c), that was correlated with intestinal EROD activity ($r = 0.62$, $p > 0.05$), but not with hepatic EROD activity. Taken together these results indicate that, probably, dietary BaP metabolites formed in the intestine were reabsorbed into the blood for enterohepatic circulation and then excreted in the bile, as shown by Kleinow et al., 1998. Although we cannot compare the real amount of BaP that is metabolized under each one of the routes of exposure, it seems also important to highlight the fact that, levels of BaP metabolites were much more elevated after water exposure (achieving a fold increase of 356 times over solvent) than after dietary exposures (achieving a fold increase of 10 times over the solvent), despite the very high levels of BaP used after both exposures routes. These results suggest that, in field studies, the levels of biliary metabolites may give an indication of the main route of exposure to the contaminant.

Results obtained for phase II enzyme GST were not very clear, since in the case of changes in activity after BaP exposure, only slight variations were seen. The data available in the literature regarding GST use as a biomarker of exposure to pollutants is not very consistent, since some authors reported increases in this enzyme activity, while others did not observe any changes or even reported considerable reductions in its activity after exposure to PAH contaminants (reviewed in van der Oost et al., 2003). Induction of GST activity was reported after waterborne exposure to BaP of *P. microps* (Vieira et al., 2008) and *S. marmoratus* (Wu et al., 2007). When it comes to dietary exposures to contaminants, some authors reported inductions of intestinal GST activities, but no induction of hepatic GST (Van Veld et al., 1991; James et al., 1997), suggesting a complementary role with phase I EROD in biotransformation of contaminants. Our findings corroborate the idea that phase II enzyme GST with CDNB as substrate has low sensibility to the presence of BaP, and should not be applied *per se*, as a biomarker of exposure to this pollutant. However, on the basis of substrate specificity, immunological cross reactivity and protein sequence data, mammalian citosolic GSTs have been grouped into seven classes: Alpha, Mu, Pi, Theta, Sigma, Omega and Zeta (Hayes et al., 2005) and previous publications have considered that the GST isoforms have distinct affinities towards the substrate CDNB (Martinez-Lara et al., 1996; Hoarau et al., 2002). Moreover, some studies have shown that, in fish, different responses are seen in GST isoforms after exposure to xenobiotics (Martinez-Lara et al., 1996; Pérez-Lopez et al., 2002; Kim et al., 2010), with some showing inducing patterns, while others do not. Therefore it is possible that, if some isoforms were upregulated and others downregulated due to BaP exposure, the net result could be no change on total GST activity, which can possibly mean that the lack of changes in GST activity after BaP exposure does not necessarily indicate that this compound had no effect on GST activity.

In conclusion, this study has shown that the disposition and effects of BaP in biotransformation pathways in Nile tilapia depend on the route of exposure to the contaminant. Waterborne exposure to BaP resulted in an induction of phase I enzyme EROD in liver, gill and intestine, while in dietary exposure route induction of EROD was only seen in intestine, and after exposure to the highest concentrations of the pollutant. Therefore, EROD activity is a reliable biomarker of exposure to BaP in Nile tilapia and, besides liver, barrier tissues, like gills and intestine, should also be considered in biomonitoring studies. BaP metabolites are good reflectors of exposure to BaP, despite the route of exposure, and the levels of metabolites can also be indicative of the route of exposure, since water exposure leads to much higher levels of the metabolites than dietary exposure. The activity of phase II enzyme GST with CDNB does not seem as a reliable biomarker of exposure to BaP regardless the route of exposure.

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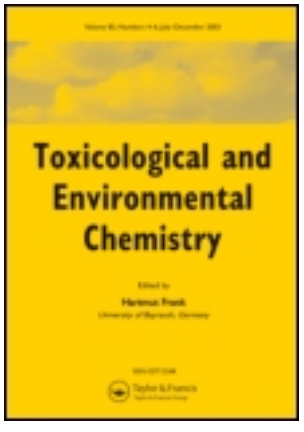

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Evaluation of 3-hydroxy-benzo[*a*]pyrene levels in Nile tilapia (*Oreochromis niloticus*) after waterborne exposure to Benzo[*a*]pyrene

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3-Hydroxy-benzo[*a*]pyrene (3-OH-BaP), a toxic compound with the ability to covalently bind with the macromolecules (proteins and DNA), is one of the major phenolic metabolites of benzo[*a*]pyrene (BaP). The purpose of this study was to evaluate the presence of 3-OH-BaP in the bile and plasma of Nile tilapia by HPLC with fluorescence detection, after waterborne exposure to BaP (10 and 100 $\mu g\,L^{-1}$). Metabolites were detected in bile and plasma, and conjugates of 3-OH-BaP (glucuronide and/or sulphate conjugates) were the majority forms in both biological fluids, glucuronide 3-OH-BaP being the main metabolite in bile. Our data suggest that extrahepatic tissues as intestine or gill are important in BaP metabolism and need to be the considered sources of metabolites released into the blood. Although, low levels of 3-OH-BaP in toxic form (free form) were detected in plasma, one cannot exclude the possibility of circulating the levels leading to adverse effects.

Keywords: PAHs; metabolites; plasma; fish; bile; HPLC-fluorescence

Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous contaminants present in the marine environment as a result of natural sources, such as incomplete combustion of organic material, but mainly arising from human activities, as uncontrolled spills, river transport, surface runoff, and atmospheric deposition (Latimer and Zheng 2003). Benzo[*a*]pyrene (BaP), one of the 16 PAH selected by the US Environmental Protection Agency (EPA) as priority pollutants, is probably the most thoroughly studied and the most common representative of this class of contaminants due to its carcinogenic and mutagenic properties (ATSDR 1995; IPCS 1998).

Fish absorb BaP through the body surface, gills, and by the ingestion of contaminated sediment or food being distributed to tissues by blood flow (Di Giulio and Hinton 2008; Fragoso, Hodson, and Zambon 2006; Varanasi, Stein, and Nishimoto 1989). Upon exposure, fish have a well-developed system that rapidly metabolizes BaP in two phases (Varanasi, Stein, and Nishimoto 1989), in a complex mixture of quinones, phenols, dihydrodiols, triols, and tretols (Kennedy and Tierney 2008; Willett et al. 2000;

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Zhu et al. 2008). In phase I, BaP is metabolized into hydroxylated derivatives (OH-BaP) by the multienzymatic system cytochrome P450 (CYP450), CYP 1A1 isozyme being the predominant, although 1A2, 2A1, and 1B1 can also be active to a minor extent (Kim et al. 1998). In phase II, the OH-BaP metabolites are conjugated with polar endogenous constituents such as glucuronic acid, sulphate, or glutathione by UDP-glucuronyl transferase, sulfotransferase, and glutathione-S-transferase, respectively, to produce water-soluble conjugates that are easily excreted by fish (Ferreira et al. 2010; Tuvikene 1995). BaP metabolites produced in the liver are secreted into the bile, concentrated and stored in the gallbladder before excretion into the intestine (Ferreira, Moradas-Ferreira, and Reis-Henriques 2006; Wang et al. 2008; Zhu et al. 2008). Although the liver is a major site of biotransformation of BaP, metabolism can also occur in extrahepatic tissues such as intestine, gills, and kidney (Costa et al. 2011; James et al. 1996; Kleinow et al. 1998; Lemaire et al. 1990). Reactive metabolites produced in these tissues can not only be transferred to the blood for elimination but can also be re-distributed to other organs, interfering with their functions and, as a consequence, producing toxicity.

BaP itself is a relatively inert molecule, but their electrophilic metabolites are toxic, mutagenic, with the ability to covalently bind with the macromolecules, such as proteins and DNA (Cachot et al. 2004; Fertuck, Matthews, and Zacharewski 2001; James et al. 1991). One of the major phenolic metabolites of BaP formed in many species, including fish, is the 3-hydroxy-benzo[*a*]pyrene (3-OH-BaP) which is produced in the liver (Wang et al. 2008; Zhu et al. 2008) and intestine (James et al. 2001). Due to the toxic features of 3-OH-BaP, it was of interest to further study the toxicity including the distribution to fish tissues. Although the blood plays an important role in the distribution of chemicals to organs, there is a lack of studies that measure BaP and metabolites in this tissue. Recently, BaP metabolism in extrahepatic tissues was confirmed in Nile tilapia (Costa et al. 2011), as evidenced from the presence of metabolites in blood.

Nile tilapia has been used in several toxicological studies and is considered a reliable model species to assess the influence of pollutants on biological systems (Coimbra, Figueiredo-Fernandes, and Reis-Henriques 2007; Pereira Trídico et al. 2010). The main aim of this study was to determine the presence of BaP and the metabolite, 3-OH-BaP, free and conjugated, in the plasma and bile of Nile tilapia (*Oreochromis niloticus*) after waterborne exposure to BaP. Possible correlations with biotransformation enzymes in the liver, intestine, and gill, were also assessed.

Material and methods

Chemicals and materials

Benzo[*a*]pyrene (99% purity), 3-hydroxy-benzo[*a*]pyrene, *tert*-butyl hydroquinone (97% purity), 4-methylumbelliferone, β-glucuronidase from bovine liver and uridine 5′-diphosphoglucuronic acid triammonium salt were purchased from Sigma Aldrich (Spain). β-Glucuronidase/aryl sulfatase from *Helix pomatia* was supplied by Merck (Germany) and Sep-Pak Plus C18 cartridges were obtained from Waters (USA). All other chemicals were of analytical grade and were purchased from local companies.

Animals and exposure experiment

Nile tilapias used in this study were born and raised at CIIMAR, Porto, Portugal. Prior to the experiments, animals ($N = 112$, average weight 70.3 ± 23 g) were randomly distributed

($n = 10$) in the experimental aquaria (52 L) and subjected to an acclimation period of 1 week before the addition of the contaminant. All tanks were supplied with biological filtration, and continuous aeration to maintain dissolved oxygen near saturation. Dechlorinated tap water was used at a temperature of $21 \pm 1°C$ with a 12 h (light:dark) photoperiod.

After the acclimatization period, fish were exposed to nominal water concentrations of 10 and 100 μg of BaP L^{-1} for 14 days. BaP stock solutions (1.04 and 5.20 g L^{-1}, respectively) were prepared in acetone and were administered directly into the aquaria. The solvent concentration never exceeded 0.002%. A solvent control group (acetone alone) was also used. Experiment was conducted under semi-static conditions and daily 80% of the water was renewed, followed by the addition of fresh BaP solution or solvent to each one of the treatment groups. Animals were fasted for 48 h before each sampling, performed at 7 and 14 days.

Sampling

Water samples were collected, in triplicate, at the beginning (0 day) and at the end of the experiment (14 days) from two aquaria, at 0, 8, and 24 h after the addition of the contaminant, for both BaP nominal concentrations.

Fish were anesthetized in ice cold water and blood was collected from the caudal vein using heparinized syringes. After blood collection, animals were sacrificed by decapitation. The blood was centrifuged at $4000 \times g$ for 7 min at 4°C and the plasma obtained was stored at −80°C until analysis. The liver, intestine, and gills were excised from the animal, and bile was collected from the gall bladder, immediately frozen in liquid nitrogen, and stored at −80°C until further use.

Biochemical analysis

Microsomal fractions of the liver, intestine and gills were prepared as described in Fernandes, Porte, and Bebianno (2007). Briefly, tissues were homogenized in ice cold 100 mM KH_2PO_4/K_2HPO_4 (pH 7.4), containing 150 mM KCl, 1 mM dithiothreitol, 0.1 mM PMSF, and 1 mM Na_2EDTA. Homogenates were centrifuged at $12{,}000 \times g$ for 20 min and the resulting supernatant was further centrifuged at $100{,}000 \times g$ for 60 min, to obtain the microsomal fractions. Microsomal pellets were resuspended in a ratio of 0.5 mL buffer g^{-1} tissue in homogenization buffer containing 20% glycerol.

UDP-glucuronyl transferase (UDPGT) activity was assayed according Collier et al. (2000). Fifteen μL 20 mM uridine 5′-diphosphate glucuronic acid was added to a reaction mixture containing 15 μL microsomal fraction and 120 μL 125 μM 4-methylumbelliferone (4MU) in 0.1 M Tris-HCl containing 5 mM $MgCl_2$ and 0.05% BSA (pH 7.4) and incubated for 10 min at 37°C. Fluorescence was measured at $\lambda_{EX} = 355$ nm/$\lambda_{EM} = 586$ nm. Activities were expressed as pmol $min^{-1} mg^{-1}$ protein using a standard curve generated with 4MU (0.031–2.50 μM). Protein content in all assays was determined by Lowry et al. (1951) method, in the correspondent fraction.

Determination of BaP

The pre-analytical treatment for the determination of BaP in water was performed according to the procedure for the extraction of PAH in water previously described by

Cheikyula et al. (2008), based on liquid–liquid extraction with *n*-hexane followed by HPLC analysis under the conditions described below.

For the analysis of BaP in bile and plasma, an analytical procedure similar to BaP extraction procedure in blood developed by Singh et al. (2008) was carried out. Bile (0.05 mL) and plasma samples (0.5 mL) were subjected to three consecutive ultrasound-assisted solvent extractions (UASEs) with 1 and 3 mL *n*-hexane, respectively, for 15 min each and the upper organic layer was collected and dehydrated with anhydrous sodium sulfate. The *n*-hexane extracts were evaporated to dryness under a gentle steam of nitrogen and the residue obtained was re-dissolved in methanol (0.1 and 0.2 mL for bile and plasma, respectively) and filtered through PTFE membranes (0.45 μm) for analysis. Pooled samples were used (2–8 fish per pool, depending on the volume of blood collected from each fish) for the determination of BaP in plasma, while for bile, individual samples were analyzed.

Determination of free and conjugates of 3-OH-BaP

The measurement of 3-OH-BaP in bile was performed according to the method of Ruddock, Bird, and McCalley (2002), which included a prior enzymatic hydrolysis of the glucuronide and sulfate conjugated metabolites with a mixture of β-glucuronidase and aryl sulfatase (30 and 60 U mL^{-1} activity, respectively) or β-glucuronidase alone (1624 U mL^{-1}). Briefly, 10 μL bile and 10 μL enzyme solution were added to 230 μL ultrapure water and the mixture was incubated at 37°C. After 45 min, the reaction was stopped by the addition of 250 μL chilled methanol. After centrifugation (16,000 × *g* for 10 min), supernatants contain total 3-OH-BaP (T-3-OH-BaP) (conjugated and free 3-OH-BaP) after β-glucuronidase/aryl sulfatase hydrolysis or a mixture of glucuronide 3-OH-BaP conjugate (G-3-OH-BaP) and free 3-OH-BaP after incubation with β-glucuronidase. For the determination of free 3-OH-BaP, the analytical procedure was carried out without enzymatic hydrolysis and an extraction step was added. Bile (50 μL) was subjected to UASE with 100 μL methanol for 10 min and, finally, the extracts were also centrifuged and filtered for HPLC analysis.

In plasma samples, the pre-analytical treatment was based on procedures for the determination of T-3-OH-BaP in biological samples such as urine or milk (Rey-Salgueiro et al. 2009; Wang et al. 2005), with the extraction and enzymatic hydrolysis steps optimized. To optimize the extraction step, plasma of non-exposed animals were spiked with 3-OH-BaP (10 μg L^{-1}) and extracted with different solvents (acetone, methanol, methanol:ethyl acetate (50:50)). Despite that the higher efficiency of extraction was obtained using methanol:ethyl acetate (97%), acetone (95%) was selected as the extraction solvent because of its higher vapor pressure, which facilitates the extraction step by being less time consuming. Plasma samples (1 or 0.5 mL, for 10 and 100 μg L^{-1} exposure levels, respectively) were subjected to three consecutive UASE with 2 × 1 × 1 mL acetone for 5 min each and the obtained extract was centrifuged (3000 × *g*) for 3 min to facilitate the separation of the liquid fraction, which was evaporated until dryness. To extract phase II metabolites from plasma samples, an enzymatic hydrolysis was conducted with a mixture of β-glucuronidase/aryl sulfatase to obtain T-3-OH-BaP. Then, the obtained residues were filled up to a final volume of 50 mL buffer (0.10 M ammonium acetate buffer, 0.80 g L^{-1} *tert*-butyl hydroquinone and acid acetic until pH 5.5). The dissolved oxygen in the solution was then displaced with a nitrogen stream. The optimization of the enzymatic hydrolysis step was carried out directly in pooled plasma samples of exposed individuals

to 100 μg L^{-1} BaP for 14 days. Volume of enzyme (10, 20 or 40 μL) and incubation time (0–16 h) were optimized for deconjugation and 20 μL was found to be an adequate enzyme amount to de-conjugate 3-OH-BaP (data not shown). Deconjugation time was optimized by adding 20 μL enzyme solution and incubating at 37°C. Conjugated metabolites were rapidly hydrolyzed in the first 2 h of incubation and slowly until 16 h (data not shown), so an overnight deconjugation (16 h) was selected to be sufficient for the hydrolysis of 3-OH-BaP in plasma samples. The hydrolysis conditions selected were in agreement with Fan et al. (2006) and Lutz et al. (2006), to enable the hydrolysis of conjugated 3-OH-BaP in urine and milk samples, respectively. Afterwards, 5 mL methanol was added and the mixture was loaded onto a C18 sep-pack cartridge (previously activated with 5 mL methanol followed by 10 mL ultrapure water). Before the elution step with 10 mL methanol:ethyl acetate (50/50), the minicolumns were dried for 15 min under nitrogen stream at 15 bars. The eluate obtained was evaporated to dryness, re-dissolved in 0.2 mL methanol and filtered for HPLC analysis. For the determination of the free 3-OH-BaP, the analytical procedure was carried out without enzymatic hydrolysis. Pooled samples were used (2–8 fish per pool) for the determination of free 3-OH-BaP and T-3-OH-BaP in plasma. The number of animals per pool was dependent on the volume of blood collected from each fish. As the available blood sample was in small amount, no G-3-OH-BaP was detected in plasma.

Methods validation

To validate the analytical methods which were modified or optimized, plasma of non-exposed animals were spiked with BaP or 3-OH-BaP and were processed ($n = 5$). The set of samples analyzed was processed with a blank to test for the background BaP and 3-OH-BaP levels in the material. Similar recovery rates of 3-OH-BaP, 59% and 60%, with RSD lower than 6.8%, were obtained at two spiked concentration levels of 1 and 10 μg L^{-1}, respectively, for the analytical method to measure 3-OH-BaP in plasma (Table 1). Other methods based on solid phase extraction showed similar, 60–66% (Fan et al. 2006), and lower recoveries, $< 50\%$ (Wang et al. 2005), in urine samples. Fan et al. (2006) suggested that this % 3-OH-BaP is not due to the method, but due to the instability of the compound in the acetate buffer, though it is stable in methanol. Therefore, the selected method was

Table 1. Recoveries ± RSD, instrument linear dynamic ranges, determination coefficients (r^2), and limits of detection (LOD) and quantification (LOQ) in μg L^{-1} ($n = 5$).

				Instrument linearity			
Compounds	Matrix	LOD	LOQ	Standards[a] concentrations range	r^2	Added (μg L^{-1})	Recovery[b] ± RSD (%)
BaP	Bile	0.83	2.50	2.5–50	0.999	5.0	96.8 ± 4.6
	Plasma	0.33	1.00	2.5–50	0.999	5.0	89.9 ± 6.0
3-OH-BaP	Bile	0.17	0.50	0.5–50	0.999	1.0	98.1 ± 5.5
	Plasma	0.07		0.5–50	0.999	1.0	59.3 ± 6.0
						10	60.4 ± 6.8

Notes: [a]($n = 12$; 7 levels in μg L^{-1} methanol in duplicate).
[b]($n = 5$ determinations).

robust enough to quantify 3-OH-BaP in plasma samples. Detection and quantification limits (LOD and LOQ) were evaluated on the basis of the noise obtained with the analysis of unfortified blank samples ($n = 5$) (Table 1). LOD and LOQ were defined as the concentration of the analyte that produced a signal-to-noise ratio of 3 and 10, respectively, (ACS 1980) and were then tested experimentally by spiking blank samples at such levels. External standard calibration was used to quantify the samples by HPLC-FD technique using standard solutions of BaP and 3-OH-BaP (Table 1).

Chromatographic conditions

The liquid chromatographic system used was a Hitachi LaChrom Elite HPLC, which constituted an L-2130 quaternary pump and an in line degasser, a L-2485 FL detector (FD), and an L-2200 autosampler. Separations were performed with a 250×4.6 mm (length $\times$ i.d.), 5 μm particle, Purospher® STAR RP-18 e analytical column obtained from Merck (Germany), and a 4×4 Ch mm i.d., 5 μm particle, guard column with the same packing material.

Mobile phases used, methanol (A) and water (B), were at a flow rate of 1 mL min^{-1}. The injection volume was set to 50 μL. For BaP elution, the temperature of the column was maintained at 33°C, and the following gradient was used: 95% A change to 100% A in 5 min, hold for 10 min, change to 95% A in 1 min, and, finally, hold for 10 min giving an analysis time of 26 min. For 3-OH-BaP elution, the temperature of the HPLC column was kept constant at 40°C and the gradient was: 70% A for 5 min, change to 95% A in 1 min, hold for 10 min, change to 70% A in 1 min, and, finally, hold for 10 min. Total run time was 27 min per sample. The excitation and emission wavelengths for BaP and 3-OH-BaP detection, were 296/406 and 308/432, respectively (Rey-Salgueiro et al. 2008).

Statistical analysis

Exposure time and treatment effects were evaluated by means of a one-way ANOVA for each one of the mentioned factors, followed by a multiple comparison test (Tukey's test) at a 5% significance level. Some data had to be log or sqrt transformed in order to fit ANOVA assumptions. Correlation (=r) between all the parameters measured was tested by Pearson correlation analysis. All the statistical tests were performed using the software Statistica 7 (Statsoft, Inc., 2004).

Ethics statement

The animals used in the research described in this article were treated in accordance with the Portuguese Animals and Welfare Law (Decreto-Lei no. 197/96) approved by the Portuguese Parliament in 1996.

Result

BaP concentration in water

In order to determine the real concentration of BaP in the water of experimental aquaria, BaP levels were measured at different times after the addition of chemical at 0 and 14 days of the experiment. No marked differences were observed between day 0 and 14 in any of

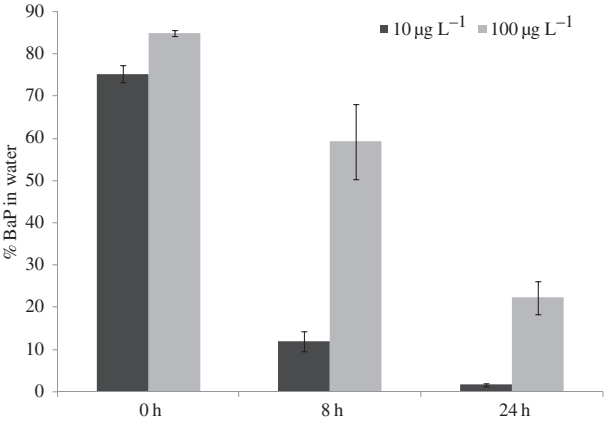

Figure 1. BaP (in percentage) in water at 0, 8, and 24 h after the addition of 10 or 100 µg of BaP L^{-1} of water. Values are presented as mean ± standard error.

the collection periods, so the results presented were combined (Figure 1). There was a fall in BaP concentrations over time at both concentrations and no BaP was detected in the solvent control group. Eight hours after the addition of BaP stock solution, there was a marked decrease in BaP, mainly at the lower concentration (88%) while for 100 µg L^{-1} it was 41%. The fall in BaP concentration was even more significant, for both concentrations, and 24 h after the addition, disappearing almost completely at lower concentration.

Bile and plasma analysis

BaP was not found in the bile and plasma of non-exposed and exposed animals. The metabolite, 3-OH-BaP, was detected in free and conjugated forms in the bile of exposed animals, as seen in Figure 2. The metabolite was not found in the bile of solvent control animals, neither as free nor as conjugated form. Free 3-OH-BaP represented 0.04 ± 0.01% and 0.03 ± 0.01% of the T-3-OH-BaP for 10 and 100 µg L^{-1} treatments (Figure 2a), respectively; thus conjugated metabolites were the major forms of 3-OH-BaP in bile, with G-3-OH-BaP as the main conjugated form present (Figure 2c). An increase in the BaP dose in water resulted in a significant increase in free 3-OH-BaP, T-3-OH-BaP, and G-3-OH-BaP levels in bile at both concentrations (Figure 2). Regarding the effect of exposure time, a significant rise in free 3-OH-BaP after 14 days exposure to higher BaP concentration was detected in comparison to 7 days. However, no significant differences were observed between days within the same treatment for T-3-OH-BaP and G-3-OH-BaP.

In plasma, only one pooled sample contained measurable levels of free 3-OH-BaP (0.4 ng mL^{-1}) from the longer exposure period to the higher BaP exposure levels. T-3-OH-BaP concentration in animals exposed to 10 µg L^{-1} was near the detection limit and the highest levels were quantified in fish exposed to100 µg L^{-1} (Figure 3). In contrast to bile, there was a significant increase of T-3-OH-BaP at 14 days after exposure to higher BaP concentration in comparison to 7 days. The levels of free 3-OH-BaP ($r = 0.73$), T-3-OH-BaP ($r = 0.89$), and G-3-OH-BaP ($r = 0.88$) in bile were significantly correlated with T-3-OH-BaP levels measured in plasma

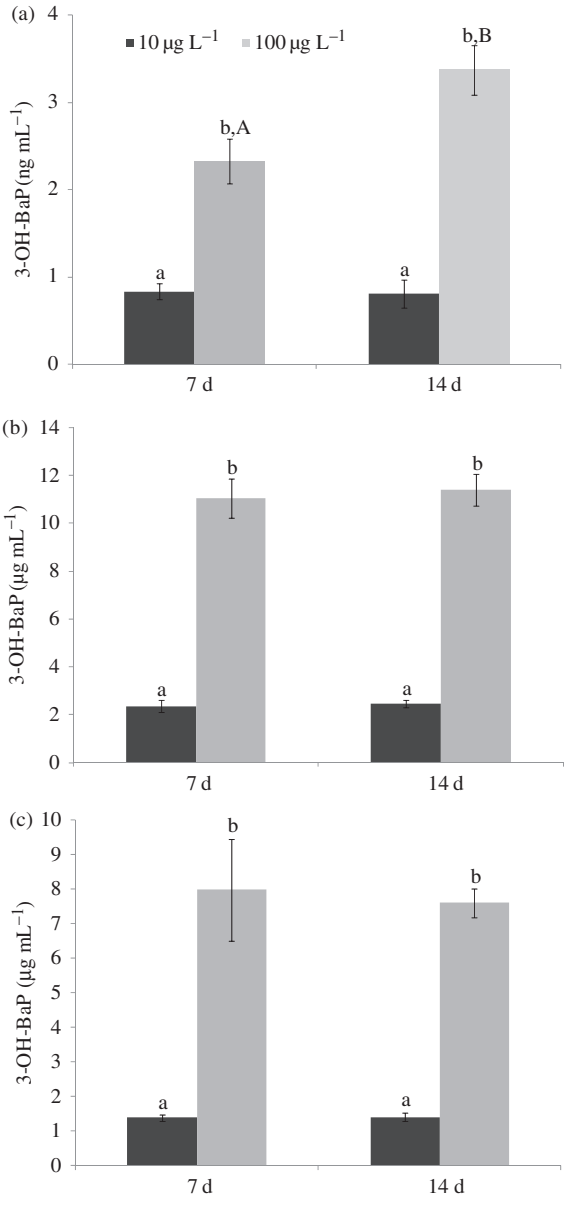

Figure 2. Free (a), total (b) and glucuronide (c) 3-OH-BaP levels in bile after waterborne exposure to BaP (10 or 100 µg L^{-1}) for 7 and 14 days. Values are shown as mean ± standard error. Different lowercase letters denote significant differences ($p < 0.05$) between groups within the same day of exposure. Different capital letters denote significant differences ($p < 0.05$) between days within the same treatment.

UDPGT activities and correlations between biotransformation enzymes and 3-OH-BaP levels

UDPGT displayed mean activities in control groups of 833.35 ± 37.9, 528.36 ± 72.78, and 794.52 ± 92.62 pmol min^{-1} mg^{-1} protein in liver, intestine and gill, respectively.

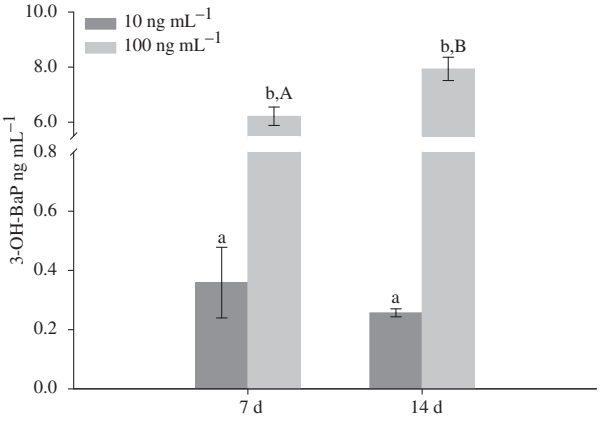

Figure 3. Total 3-OH-BaP levels in plasma after waterborne exposure to BaP (10 or 100 μg L^{-1}) for 7 and 14 days. Values are shown as mean ± standard error. Different lower case letters denote significant differences ($p < 0.05$) between groups within the same day of exposure. Different capital letters denote significant differences ($p < 0.05$) between days within the same treatment.

UDPGT activity in exposed animals was not markedly different from solvent control in any of the tissues analyzed; however, a significant increase in UDPGT activity was seen from 7 to 14 days at 10 μg L^{-1} in intestine and gill. UDPGT activity at 100 μg L^{-1} exposure was significantly lower than at 10 μg L^{-1} at 14 days in intestine and gill (Table 2).

Nile tilapia EROD and GST activities in liver, intestine and gills after waterborne exposure to BaP (10 or 100 μg L^{-1} were determined by Costa et al. (2011) and UDPGT activities measured in this study are presented in Table 2. Positive statistically significant correlations were observed between T-3-OH-BaP concentrations in plasma and EROD activities in the liver ($r = 0.51$), intestine ($r = 0.73$), and gills ($r = 0.76$). The same was also observed between free 3-OH-BaP level in bile and EROD activities in intestine ($r = 0.71$) and gill ($r = 0.59$). In contrast, the free metabolite was not correlated with this enzyme activity in liver. Positive statistically significant correlations were obtained between T-3-OH-BaP concentration, in plasma and in bile, and GST activity in the liver ($r = 0.82$ and $r = 0.68$, respectively) but, were not correlated with activity in intestine and gill.

Discussion

The contamination of aquatic ecosystems by organic contaminants as PAH is a matter of great concern in environmental toxicology. BaP is one of the most common and toxic PAH, and its toxicity is mainly linked to the capacity of fish species to biotransform the compound into reactive metabolites. This study aimed at evaluating BaP metabolism into 3-OH-BaP, a toxic metabolite, and the metabolite bioavailability in bile and plasma of Nile tilapia after waterborne exposure.

The real concentration of BaP detected in the water from the experimental aquaria was significantly lower than the nominal concentration (Figure 1). The decrease of BaP concentration observed in the water is in agreement with studies where the stability of BaP was evaluated in similar conditions as the ones in our study, but without fish (Swietlik, Kowalczyk, and Dojlido 2002); the higher decrease of BaP observed in our experiment

Table 2. EROD, GST, and UDPGT activities in liver, intestine, and gill of Nile tilapia after waterborne exposure to BaP (10 and 100 $\mu g\,L^{-1}$).

		EROD[a]		GST[a]	UDPGT		
Tissue	BaP dose ($\mu g\,L^{-1}$)	7 d	14 d	7 d	14 d	7 d	14 d
Liver	10	310.49 ± 57.17^{b}	222.59 ± 37.75	93.82 ± 5.17	90.64 ± 6.44	99.35 ± 12.98	83.23 ± 10.19
	100	744.44 ± 144.38^{b}	504.91 ± 96.80^{b}	113.14 ± 12.98	139.20 ± 5.71^{b}	106.73 ± 13.61	74.70 ± 13.98
Intestine	10	510.15 ± 248.45	624.32 ± 183.13	77.90 ± 29.01	62.48 ± 5.29	73.37 ± 14.75^{c}	$124.15 \pm 18.48^{d,e}$
	100	1586.73 ± 790.71^{b}	2372.01 ± 307.26^{b}	68.97 ± 14.43	35.81 ± 2.83^{b}	81.96 ± 17.25	73.37 ± 5.14^{f}
Gill	10	643.74 ± 29.77^{b}	799.58 ± 91.49^{b}	97.99 ± 9.57	119.56 ± 24.14	92.64 ± 8.13^{c}	$132.92 \pm 12.98^{d,e}$
	100	1320.66 ± 378.50^{b}	1738.96 ± 196.69^{b}	166.13 ± 14.96^{b}	118.21 ± 13.52	82.71 ± 10.77	79.76 ± 11.15^{f}

Notes: Values are expressed in percent of activity ($pmol\,min^{-1}\,mg^{-1}$ protein for EROD and UDPGT; $nmol\,min^{-1}\,mg^{-1}$ protein for GST) in relation to solvent control (set to 100%), and are shown as mean ± standard error.
[a]Costa et al. (2011).
[b]Significant differences ($p < 0.05$) in comparison to solvent control.
[c,d]Significant differences ($p < 0.05$) between days within the same treatment.
[e,f]Significant differences ($p < 0.05$) between groups within the same day of exposure.

could be explained by the presence of fish. Moreover, due to the same number of animals in aquaria (at both concentrations), a higher decline of BaP was also observed at the lower concentration.

Fish have a high capacity to metabolize PAH, thus no BaP was detected in bile and plasma of Nile tilapia and only BaP metabolites (free and conjugated) were detected. Hence, our results show the uptake and the ability of Nile tilapia to metabolize BaP and neutralize the toxic metabolite, which demonstrates a well-developed biotransformation system, eliminating the most toxic forms of BaP (free 3-OH-BaP) into non-toxic forms (conjugated form). In bile, the predominance of conjugates over free metabolites, mainly G-3-OH-BaP, was consistent with results observed in *Fundulus heteroclitus* after waterborne exposure at the same concentrations of BaP (Zhu et al. 2008). In different exposure conditions, by single intraperitoneal injection ($10\,mg\,kg^{-1}$), Ictalurid catfish bile was found to have also a higher % biliary G-3-OH-BaP (Willett et al. 2000). Contrary to the observed dose-dependent response, time-dependence responses were observed only with free 3-OH-BaP in bile. Comparable results, upon waterborne exposure to BaP, were reported in *Sparus macocephalus* (Wang et al. 2008) and in sole (*Solea solea*), after dietary exposure to an equimolar mixture of BaP, pyrene, and fluoranthene (Wessel et al. 2010). In contrast, when measuring total BaP metabolites as fluorescent aromatic compounds (FAC) in bile by fixed wavelength fluorescence, instead of the HPLC analysis, as in the previous studies (including this one), significant time-dependent responses after BaP waterborne exposure were reported (Boleas et al. 1998; Costa et al. 2011). The assessment of FAC in bile gives the total metabolites and not specific ones, so analytical techniques, like HPLC, that allow the measurement of individual compounds yield more precise information regarding PAH metabolism, especially when it applies to free metabolites and conjugation.

In plasma, free 3-OH-BaP was detected in some plasma samples, but quantification was only possible in one pooled sample of animals exposed to higher BaP concentration for 14 days ($0.4\,ng\,mL^{-1}$). The low concentrations of free 3-OH-BaP detected in plasma might be due to its propensity to bind with the components of the blood and form unextractable products (James et al. 2001), or due to the rapid phase II conjugation in liver and extrahepatic tissues (James et al. 1996). T-3-OH-BaP metabolite was detected and the levels were considerably lower than the ones detected in bile ($0.3 \pm 0.04\,ng\,mL^{-1}$ *vs.* $2.5 \pm 0.1\,\mu g\,mL^{-1}$ in $10\,\mu g\,L^{-1}$ at 14 days).

In this study, despite the high G-3-OH-BaP levels detected in bile, no induction of UDPGT was observed in liver, intestine, or gill. Even though an increase in UDPGT activity over time was noted at lower BaP exposure levels in intestine and gill, the lack of UDPGT induction by BaP in Nile tilapia in this study is in agreement with the low sensitivity of this enzyme that was reported in fish collected in field (Della Torre et al. 2010; Schreiber, Otter, and Van Den Hurk 2006). Moreover, after exposure via intraperitoneal injection (10 or $50\,mg\,kg^{-1}$), similar results were found in longear sunfish (*Lepomis megalottis*) (Brammell et al. 2010). In a previous study, EROD and GST induction activities were shown in liver, intestine, and gill of Nile tilapia after BaP exposure under similar experimental conditions (Costa et al. 2011). A joint analysis of the results obtained in this study and those previously published showed positive correlations between enzymatic activities and free and T-3-OH-BaP levels in bile and plasma. The gill and intestine EROD induction (Costa et al. 2011) and the positive correlations between enzymatic activities and T-3-OH-BaP concentration in plasma and free 3-OH-BaP in bile strengthen the notion that these extrahepatic tissues are involved in the metabolism of BaP, and that the metabolites formed are released into the blood stream. Moreover, phase

II enzymes activity (GST and UDPGT) in intestine and gill and the predominance of conjugates metabolites in plasma corroborate that these extrahepatic tissues also play an important role in the elimination of the toxic form of 3-OH-BaP. Taken together, these results indicate that liver and extrahepatic tissues such as intestine and gill may be important sources of metabolites into the blood but in addition, they work together to reduce the circulating levels of free 3-OH-BaP.

The major concern regarding the exposure to BaP is the adverse effects produced by the presence of the toxic form of 3-OH-BaP. Some studies evaluated the *in vitro* 3-OH-BaP effects and showed that low concentrations of 3-OH-BaP (from 8 to 67 ng mL^{-1}) may exert estrogenic activity (van Lipzig et al. 2005) and form covalent bonds with macromolecules (Moorthy et al. 2003; Sugihara and James 2003), leading to adverse consequences at the organism level. Although adverse effects were not assessed and the levels of free 3-OH-BaP measured in plasma (0.4 ng mL^{-1}) were lower than concentrations capable of producing toxic effects, as it was quantified in one pooled sample, the levels obtained in our study may have been diluted. Hence, individual fish may have higher concentrations than the ones determined and as a consequence exposed to adverse outcomes from this reactive form of 3-OH-BaP.

Conclusions

This study showed that the capacity of Nile tilapia to metabolize BaP resulted in high concentrations of metabolites in bile and plasma. The predominance of conjugated over free forms of 3-OH-BaP in bile and plasma indicated the importance of phase II in metabolism and elimination of BaP in Nile tilapia. Our data suggest the presence of extrahepatic metabolism of BaP which may be responsible for the presence of 3-OH-BaP metabolite in plasma. Although low levels of free 3-OH-BaP, reactive form, were detected in plasma, one can not exclude the possible adverse effects to Nile tilapia.

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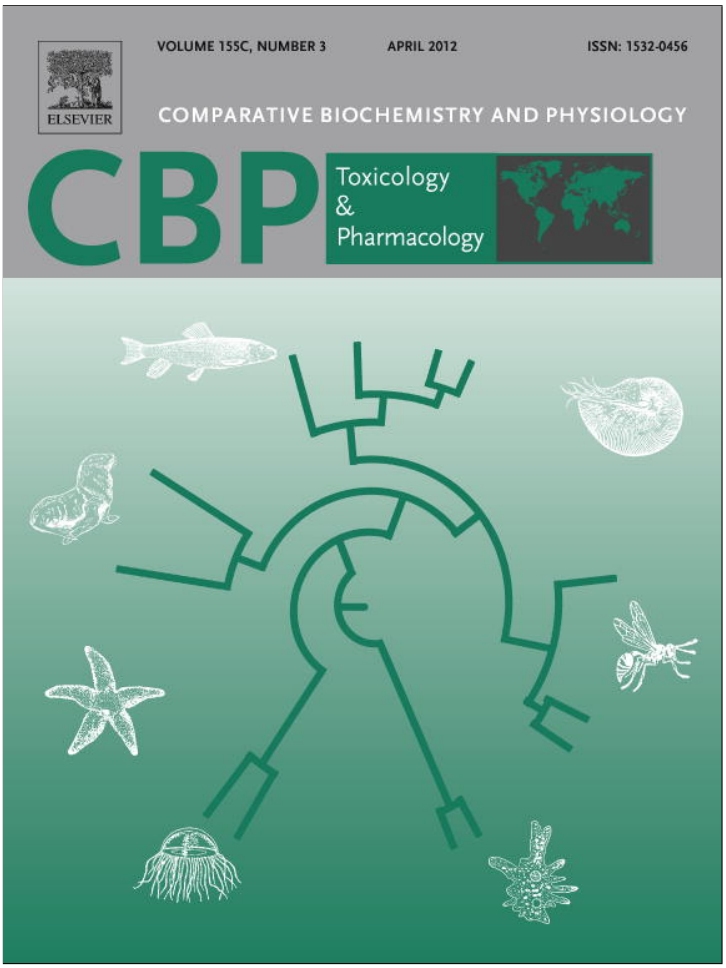

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Gene expression analysis of ABC efflux transporters, CYP1A and GSTα in Nile tilapia after exposure to benzo(a)pyrene

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ABSTRACT

The aim of this study was to evaluate the response of ABC transporters, CYP1A and class alpha (α) GST genes, upon water and dietary exposures to benzo(a)pyrene (BaP) in *Oreochromis niloticus*. Partial mRNA sequences of ABC transporters (ABCB1b, ABCB11, ABCC1, ABCC2 and ABCG2) were identified, and their tissue distribution patterns evaluated in liver, gill and intestine, showing similarities with other fish and mammals. After 14 days of water exposure to BaP, ABC transporters mRNA expression was up-regulated, namely ABCC2 in gill (up to 16-fold) and ABCG2 in liver (up to 2-fold) and proximal intestine (up to 7-fold). CYP1A mRNA expression was up-regulated in water exposed animals, with maximum fold inductions of 5, 35 and 155, respectively in liver, gill and proximal intestine. After dietary exposure, intestinal CYP1A mRNA showed a 13-fold increase in exposed animals. No significant changes were seen in ABCB1b, ABCC1 and GSTα mRNA expression after both routes of exposure to BaP. In conclusion, this study has shown that transcriptional expression of some ABC transporters and CYP1A respond to the presence of BaP, indicating a possible involvement and cooperation in the detoxification process in Nile tilapia.

1. Introduction

The ATP binding cassette (ABC) superfamily is a highly conserved family of transmembrane proteins (Dean and Annilo, 2005), and some of its members confer Multixenobiotic Resistance (MXR) in aquatic organisms by acting as efflux pumps of a wide variety of putative toxicants, and/or its metabolites (Kurelec, 1992). MXR is similar to a previously described phenotype of Multi-Drug Resistance (MDR), first observed in mammalian tumor cells (Gottesman et al., 1996), and related to the over expression of an ABC transporter (ABCB1) responsible for the efflux of a high number of anti-cancer agents (Chan et al., 2004). In addition to ABCB1 (P-glycoprotein, Pgp), studies using various animal models, have indicated ABCCs 1–5 (Multiresistance Associated Proteins, MRPs) and ABCG2 (Breast Cancer Resistance Associated Protein, BCRP) as the most relevant in the toxicological context (reviewed in Leslie et al., 2005). Another transporter from the ABCB family, ABCB11 (Bile Salt Export Pump, BSEP), has also been studied in aquatic organisms (Zaja et al., 2008a; Zaja et al., 2008b; Loncar et al., 2010) mostly due to its high degree of similarity with ABCB1, since mammalian studies indicate that ABCB11 exports bile salts from hepatocytes into bile canaliculus, and is not involved in the efflux of xenobiotics (Gerloff et al., 1998). In aquatic toxicology, research on these transporters has been mainly focused on ABCB1 (Kurelec, 1992; Bard et al., 2002a; Bard et al., 2002b) while the remaining ABC transporters are less studied. Although they have been identified in different fish species (Zaja et al., 2008a; Zaja et al., 2008c; Paetzold et al., 2009; Fischer et al., 2010; Loncar et al., 2010) both at gene and protein levels, only a few studies have investigated the modulation of these transporters by environmental pollutants in fish (Bard et al., 2002a; Bard et al., 2002b; Paetzold et al., 2009; Zucchi et al., 2010; Long et al., 2011a; Long et al., 2011b).

These proteins translocate a wide variety of substances and, although there is some overlap in substrate specificity, there are differences in transport mechanisms and chemical composition of substrates. ABCB1 transports moderately hydrophobic, amphiphilic, neutral or positively charged substances, including exogenous and endogenous compounds, while ABCCs and ABCG2 primarily transport organic anions conjugated to glutathione, glucoronide, sulfate or other polar groups (Leslie et al., 2005). Considering these characteristics, it has been previously suggested that ABC transporters may be similarly regulated in a coordinated fashion with phase I and II enzymes, providing an important mean of protection to the cell from xenobiotic insults (Bard, 2000; Leslie et al., 2005; Xu et al., 2005). It is believed that ABCB1 acts as a first line of defense preventing unmodified compounds from accumulating in the cell (phase 0 of cellular detoxification), while ABCCs and ABCG2 transport phase I and II metabolites, therefore acting in phase III of cellular detoxification (Bard, 2000; Sturm and Segner, 2005). Induction of phase I cytochrome P4501A (CYP1A) and phase II glutathione *S*-transferases

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(GSTs) towards many environmental pollutants has been widely studied, including to Polycyclic Aromatic Hydrocarbons (PAHs) which are known agonists of the Aryl hydrocarbon Receptor (AhR) that controls the expression of these genes (for a review see van der Oost et al., 2003). Recent studies have evaluated the hepatic expression of some ABC transporters and phase I and/or phase II enzymes in the presence of pollutants (Paetzold et al., 2009; Della Torre et al., 2010; Zucchi et al., 2010), but the exact way of action of these putatively important parts of the cellular detoxification has not been experimentally demonstrated yet. Based on substrate specificity, immunological cross reactivity and protein sequence data, mammalian citosolic GSTs have been grouped into seven classes: Alpha (α), Mu, Pi, Theta, Sigma, Omega and Zeta (Hayes et al., 2005). GSTα is one of the most expressed classes in fish (Fu and Xie, 2006; Li et al., 2010) and has been associated with a multidrug-resistance phenotype observed in rats (Hayes and Pulford, 1995). Benzo(a)pyrene (BaP) is an common PAH, widely spread in the aquatic environments, whose carcinogenic and mutagenic properties have been extensively studied (Buhler and Williams, 1989; Tsukatani et al., 2003), and was used as a model contaminant in this study. BaP is a known inducer of phase I and II enzymes, like CYP1A and GSTs, and the transport of the parent compound and/or its metabolites by ABC transporters proteins has been hypothesized (Chao Yeh et al., 1992; Buesen et al., 2002; Bard, 2000; Lampen et al., 2004; Myllynen et al., 2007) and remains a matter of debate. Taking in account that in aquatic animals the uptake of pollutants can occur through water, or through contact with food or sediments, we have exposed the model specie Nile tilapia (*Oreochromis niloticus*) to BaP, by waterborne and dietary exposure routes. Besides of being an economically important cultured species, namely in Asia and Africa, Nile tilapia is also a well established model in many toxicological studies (Almeida et al., 2001; Straus, 2003; Coimbra et al., 2005; Figueiredo-Fernandes et al., 2006; Coimbra et al., 2007; Costa et al., 2011; Rey-Salgueiro et al., 2011).

The main objectives of this work were 1) the identification and sequence characterization of toxicologically relevant ABC transporters (ABCB1, ABCB11, ABCC1, ABCC2 and ABCG2) in *O. niloticus*, and 2) the quantification of mRNA expression of ABC transporters, CYP1A and GSTα after waterborne and dietary exposures to BaP, in tissues involved with the uptake and metabolism of pollutants, as liver, gills and intestine, in order to evaluate, at a transcriptional level, the possible correlations between ABC transporters, CYP1A and GST. Novel information is provided regarding ABC transporters identification in *O. niloticus*, and on gene expression changes upon BaP exposure in different tissues.

2. Materials and methods

2.1. Animals

Animals (*O. niloticus*) used in this study were hatched and raised in the laboratory (CIIMAR, Porto, Portugal). The corresponding breeder stock was obtained from the Aquaculture Station of UTAD (Universidade de Trás-os-Montes, Portugal). Animals were juvenile, and therefore not sexually mature, as confirmed by macroscopic analysis of the gonads when animals were sampled. Until the start of the exposure assays, fish were kept in 60 L aquaria supplied with biological filtration. Prior to the experiments, animals were randomly distributed in the experimental aquaria (30 L), and submitted to an acclimatation period of one week. All tanks were supplied with continuous aeration to maintain dissolved oxygen near saturation. Dechlorinated tap water was used at a temperature of 20 ± 2 °C, with a 12 h:12 h (light:dark) photoperiod. Fish were fed commercial food pellets (Aquasoja, Portugal), until satiation, once a day.

2.2. Stock solutions of BaP and preparation of contaminated food

For the waterborne exposures, stock solutions of BaP were prepared in acetone (0.5, 1.25 and 2.5 g/L) and were administered directly to the experimental aquaria. The percentage of solvent added to the experimental aquaria was 0.002%. For the dietary exposures a stock solution of BaP with a concentration of 40 g/L was prepared. The contaminated diets were prepared by immersion of food pellets in BaP stock solutions diluted in acetone, in a proportion of 0.32 mL/g of food. For control groups, nothing was added to the food pellets, and for solvent control groups only acetone was added to the food pellets. Acetone was evaporated under forced air current for 24 h, until the pellets were completely dry, and diets were stored at −20 °C.

2.3. Xenobiotic exposures

After the acclimatation period, fish were exposed to BaP either via water or via food. For the water exposure, juvenile Nile tilapia (N = 20, average mass 16.36 ± 0.82 g, average length 9.99 ± 0.19 cm) were exposed to nominal water concentrations of 10, 25 and 50 μg of BaP/L for 14 days. Also, a control group and a solvent control group (acetone alone) were maintained. The tested concentrations have been found in pore waters of estuaries polluted with petrochemical products (Maskaoui et al., 2002). Waterborne exposures were conducted in semi-static conditions in 30 L aquaria. Daily, 80% of the water was renewed, followed by the addition of fresh BaP solution or solvent to each one of the treatment groups. Animals were fed to satiation every two days, with the exception of the day before sampling. Sampling was performed 14 days after the contaminant addition.

For dietary exposures, fish were maintained in 30 L aquaria in continuous water flow conditions, which assured 100% of water renewal per day. Juvenile tilapia (N = 16, average mass 10.88 ± 0.52 g, average length 8.53 ± 0.16 cm) were exposed to 100 and 200 μg of BaP/g of food for 14 days. A control (uncontaminated food) and a solvent control (acetone alone) were maintained. Fish were fed daily at a rate of 3% of body weight. After feeding, animals were observed to assure that the total of the food was consumed in 2 to 3 min. Fish were sampled after14 days of exposure. The chosen levels of dietary exposure fall within a range of PAH concentrations that may be found in prey or in sediment that is ingested during feeding at heavily contaminated locations (Naes et al., 1995; Viguri et al., 2002).

2.4. BaP determination in water and food samples

To determine the real concentration of BaP in the water of the experimental aquaria, BaP was extracted from water samples according to the method described by Cheikyula et al. (2008), based on liquid–liquid extraction with n-hexane followed by HPLC analysis, under the conditions described in Rey-Salgueiro et al. (2008). After the addition of the contaminant, the real concentrations of BaP in water samples were 10.49, 22.51 and 41.46 μg/L, respectively for the nominal concentrations of 10, 25 and 50 μg/L. After 24 h, and before the addition of fresh contaminant to the aquaria, the amount of BaP still present in each one of the treatment groups was 0.34, 0.69, 12.19 and 25.70 μg/L. In water collected from control and solvent control groups no BaP was detected at both times. Food pellets supplied to the fish in dietary exposures were also analyzed in terms of BaP concentration, according to the method described by Rey-Salgueiro et al. (2009), based on ultrasound-assisted solvent extraction, followed by HPLC analysis. The real BaP concentrations in the food pellets were 0.06, 0.07, 36.24 and 92.70 μg/g, respectively for control, solvent, 100 and 200 μg/g. When referring to the concentrations of BaP used in water and dietary treatments nominal concentrations are mentioned throughout the manuscript.

2.5. Sampling

Fish were anesthetized on ice cold water and sacrificed by decapitation. Liver, gills and proximal intestine (first one third of the intestine) were excised from the animal, placed in RNA later at 4 °C overnight, and stored at −80 °C.

2.6. RNA isolation, RT-PCR, cloning and sequence analysis

Primer pairs for ABCB1b, ABCB11, ABCC1, ABCC2 and ABCG2 genes were designed based on highly conserved regions among mammalian and fish species. The oligonucleotides were purchased from Stabvida (Portugal). Total RNA from liver, gills and proximal intestine was isolated from, approximately 20 mg of tissue, using the Illustra RNAspin Mini (GE Healthcare), according to manufacturer's protocol. Total RNA was quantified through the measure of the optical density at λ260 nm, and its quality was assessed by separation in 1% agarose gel electrophoresis (in Tris-acetate-EDTA – TAE – buffer) stained with ethidium bromide, and by measurement of the ratio of the optical density at $\lambda_{260/280\ nm}$ (1.8–2.0). One microgram of total RNA was reverse transcribed to produce the first strain of cDNA (iScript cDNA Synthesis Kit, BioRad), according to the manufacturer's instructions. PCR was performed in a Biometra Thermocycler with 100 ng of cDNA (composed by a mixture of cDNA from liver, gill and proximal intestine in equal amounts), 0.2 mM of DNTPs, 0.4 μM of each primer and 2.5 mM of $MgCl_2$ in a total volume of 50 μL, using Taq DNA Polymerase (5-Prime, Inc, EUA), with the following conditions: 2 min denaturation at 94 °C, 40 cycles of denaturation for 30 s, 30 s of annealing (different annealing temperatures — Table 1) and 1 min of 72 °C of polymerization, and 10 min of final extension at 72 °C. The aliquots of the PCR reactions were separated by gel-electrophoresis on a 2% agarose gel in TAE buffer stained with ethidium bromide, and the PCR products were visualized under UV light. The bands of expected size were excised, and eluted from the gel according to manufacturer's protocol, using the commercial kit Illustra GFX Tm PCR DNA and Gel Band Purification Kit, GE Healthcare. The isolated fragments were then inserted in pGEM plasmid vector (pGEM® – T Easy Vector Systems – Promega) and incorporated in E. coli using Nova Blue Competent Cells (Novagen). After the selection of the correct colonies developed for 10 h in solid medium (35 g/L LB Broth, ampicillin 0.1 mg/mL, IPTG 0.1 mM and X-gal 100 mM) at 37 °C, the plasmids were isolated from 5 mL of overnight culture (at 37 °C) using Wizard Plus SV Minipreps DNA Purification System (Promega). The inserts were sequenced by Stabvida (Portugal), and the identities of all sequences were checked using the Basic Local Alignment Search Tool (Blast) at National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Predicted topologies for the aminoacid partial identified sequences were performed with the Polyphobius algorithm (http://phobius.sbc.su.se/poly.html). Alignments of the obtained sequences with other mammal and fish species were conducted with ClustalW Multiple Alignment (Thompson et al., 1994). Phylogenetic analysis was performed with Mega 4.0.2 (Tamura et al., 2007) using neighbor-joining method and a percentage of concordance based on 1000 bootstrap iterations.

Table 2
Primer sequences, amplicon lengths and efficiency of reaction, used in ABC transporters, CYP1A, GSTα and 18SRNA gene expression quantification by qRT-PCR in Nile tilapia.

Target gene	Sense	Antisense	Amplicon length	Efficiency of the PCR reaction
ABCB1b	cgttcctcaaggtgatggct	ggctgcattgcaccattgat	91 pb	98.5%
ABCB11	ctggtcagacactggccttt	caggaaagacacgttgacgc	143 pb	110.0%
ABCC1	atccgtgagagtgaccag	caaatgacacaatgaagtttcc	117 pb	99.7%
ABCC2	cctggttggcttgtctatatcc	ctcgctgtattcactcactctc	123 pb	107.6%
ABCG2	tcatgaagccgggtctcaac	agacctgcagggtccttttct	96 pb	103.9%
CYP1A	cgtcgtcgtctctgttgcc	catcgtcgtggtggtcatagc	70 pb	96.6%
GSTα	aaatggatggcatgaagctc	tcgttctttgggatcctttg	92 pb	109.8%
18SRNA	cggaaggatcattactggctacac	agaccctcggcggcaaag	78 pb	100.1%

2.7. Quantitative real-time PCR (qRT-PCR)

Gene expression quantification of ABCB1b, ABCB11, ABCC1, ABCC2, ABCG2, CYP1A, GSTα was performed in liver, gill and proximal intestine, by means of quantitative real time PCR (qRT-PCR). Elongation factor 1 (EF1) and 18S ribosomal RNA (18SRNA) were both evaluated as possible reference genes, and 18SRNA was chosen since its expression proved not to be affected by the treatments, and to be stable between the analyzed tissues (data not shown). For qRT-PCR, 1 μg of total RNA was subjected to digestion of genomic DNA using Deoxyribonuclease I, Amplification Grade (Invitrogen) and first strand cDNA was synthesized using the iScript cDNA Synthesis Kit from BioRad. Specific primers for ABCB1b, ABCB11, ABCC1, ABCC2 and ABCG2 were designed based on the previously partially sequenced genes in Nile tilapia, using Beacon Designer™ software. Primers for CYP1A, GSTα, 18SRNA and EF1 were designed based on partial mRNA sequences of *O. niloticus*, available on Genbank (accession numbers GI13365614 for CYP1A, EU234530 for GSTα, DQ397879 for 18SRNA and AB075952 for EF1), using the Primer-Blast software (http://www.ncbi.nlm.nih.gov). To confirm the identities of the amplicons for qRT-PCR, RT-PCR reactions were conducted and, after excision from the gel, products were cloned and sequenced (as described in section 2.6). Optimal primers concentrations were determined (600/600 nM) after evaluation of the highest fluorescence signal at lower Ct number. Primer sequences and amplicon lengths of target gene sequences are given in Table 2. Real-time PCR amplification was done in a IQ5 Bio Rad, with the use of IQ Sybr Green Supermix (Bio Rad), with 10 μL SYBR Green mix, 2 μL of each primer (6 μM) and 1 μL of cDNA in a total volume of 20 μL. Reactions were conducted under the following conditions: 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, 54 °C for 30 s and 72 °C for 30 s. At the end of each run a melting curve analysis was done (from 55 °C to 95 °C) to determine the formation of specific products.

Table 1
Primer sequences, sequence lengths, annealing temperatures, GenBank accession numbers and homology percentages with other animal species of ABC transporters identified in Nile tilapia.

Target gene	Sense	Antisense	Sequence length (bp)	Annealing temperature	Acession number	Homology with other species
ABCB1b	cctgtgtcgttcctcaaggt	cctctcttgtgaggcagac	922	56 °C	GQ911571	*Poeciliopsis lúcida* — 80%; *Oncorhynchus mykiss* — 75%; *Trematomus bernachii* — 75%; *Homo sapiens* — 63%
ABCB11	catttcagcctggtgttcag	gtcagagttctggatggtgg	820	60 °C	GQ911570	*Platichthys flesus* — 93%; *Fundulus heteroclitus* — 93%; *Oncorhynchus mykiss* — 91%; *Danio rerio* — 91%; *Homo sapiens* — 84%
ABCC1	atgttcatgggctccatgttca	ggatccaggttcatcctcaga	1062	56 °C	GQ911567	*Danio rerio* — 84%; *Oncorhynchus mykiss* — 66%; *Homo sapiens* — 75%
ABCC2	ctcagtgtatctgcagtacct	ctaaacaggcagttggtaagac	1134	56 °C	GQ911569	*Oncorhynchus mykiss* — 88%; *Danio rerio* — 86%; *Monodelphis domestica* — 71%; *Homo sapiens* — 72%
ABCG2	atcggactcaatggcatcatga	gatgaagagttcagctgctga	1100	56 °C	GQ911568	*Oncorhynchus mykiss* — 90%; *Danio rerio* 88%; *Salmo salar* — 85%; *Homo sapiens* — 73%

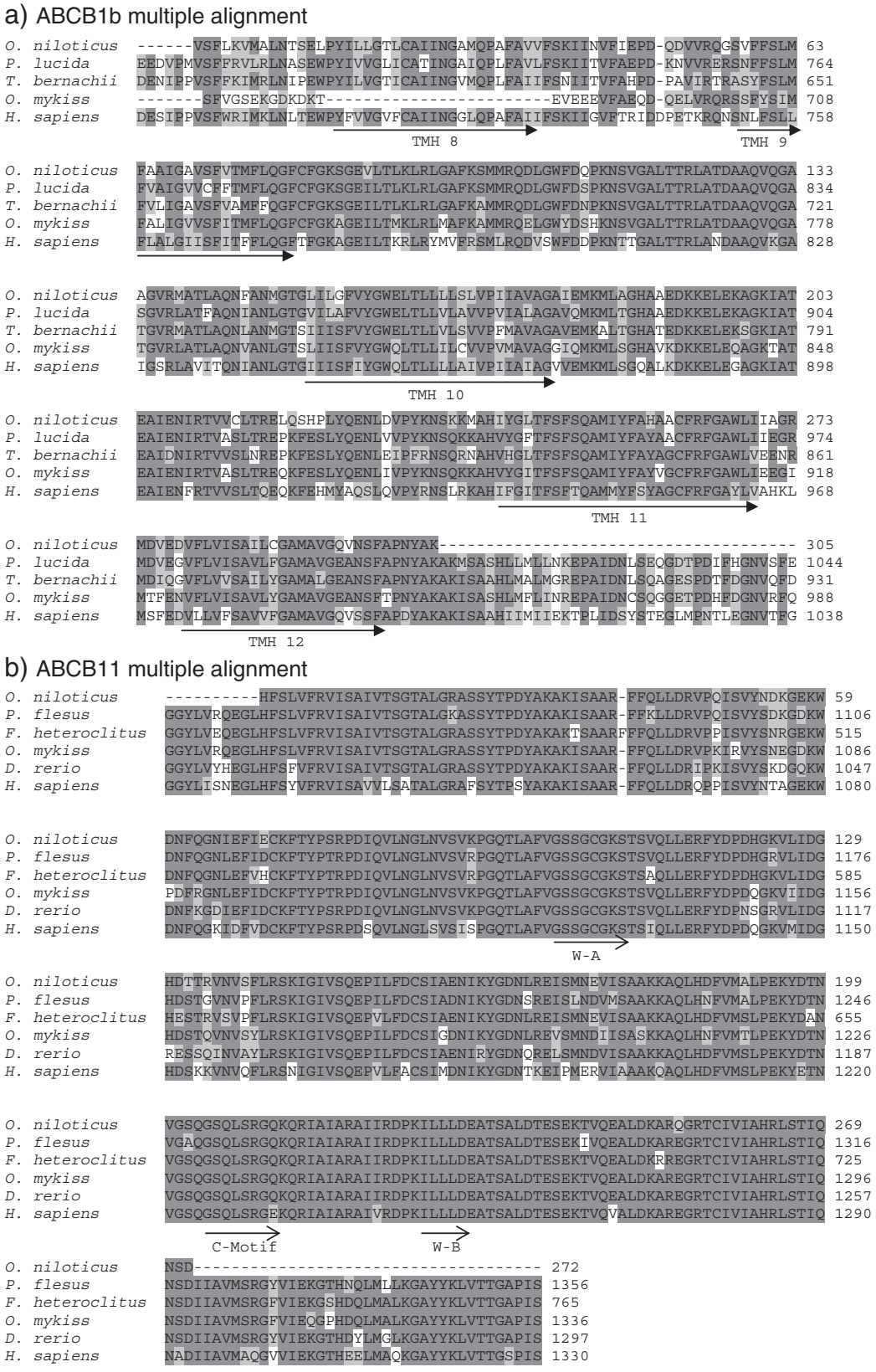

Fig. 1. Multiple alignments of the deduced amino acid sequences for ABCB1b (a), ABCB11 (b), ABCC1 (c), ABCC2 (d) and ABCG2 (e) in N. tilapia with other fish species and human. Arrows indicate transmembrane helices (TMH), and/or ABC signature regions (C-Motif), Walker-A (W-A) and Walker-B (W-B), in each partial amino acid sequence. Identical and similar amino acids are indicated in dark and light gray, respectively. ABCB1: *Poeciliopsis lucida* ADQ20481.1; *Trematomus bernachii* ACX30417.1; *Oncorhynchus mykiss* AAW56424; *Homo sapiens* NP000918.2. ABCB11: *Platichthys flesus* CAC86593.1, *Fundulus heteroclitus* AAD29692.1, *O. mykiss* NP001118128.1, *Danio rerio* XP001923538, *H. sapiens* NP003733.2. ABCC1: *D. rerio* XP001341895.3, *O. mykiss* NP001161802.1, *H. sapiens* NP004987.2. ABCC2: *O. mykiss* NP001118127.1, *D. rerio* XP002664118, *Monodelphis domestica* XP001372990.1, *H. sapiens* CAB45309.1. ABCG2: *O. mykiss* NP001118155.1, *D. rerio* NP001036240.1, *Salmo salar* NP001167126.1, *H. sapiens* AAH92408.1. Alignments were done with Clustal W program.

c) ABCC1 multiple alignment

```
O. niloticus    --GLIYFFVQRFYVSSRQLKRLESVSRSPIYTHFNETPLGTSVIRAFGEQERFIRESDQRVDHNQKAYY 68
D. rerio        PLGLLYFFVQRFYVASSRQMKRLESVSRSPVYTHFNETLLGTSVIRAFGEQQRFIKESDGRVDHNQKAYF 1187
O. mykiss       PLTLLYAFIQSFYVATSCQLRRLEAVSRSPIYTHFNETFQGASVIRAFSEQERFTLQANGRIDHNQTAYF 1124
H. sapiens      PLGLIYFFVQRFYVASSRQLKRLESVSRSPVYSHFNETLLGVSVIRAFEEQERFIHQSDLKVDENQKAYY 1075

O. niloticus    PSIVANRWLAVRLEFVGNFIVSFAALFAVIARESLSPGIMGLAISYALQLTASLTWLVRMSSDVETNIVA 138
D. rerio        PSIVANRWLAVRLEFVGNCIVTFAALFAVMARNNLSPGIMGLSISYALQVTASLNWLVRMSSELETNIVA 1257
O. mykiss       PRFVATRWLAVNLEFLGNLLVLAAATLAVMGRDTLSPGIVGLAVSHSLQVTGILSWIVRSWTDVENNIVS 1194
H. sapiens      PSIVANRWLAVRLECVGNCIVLFAALFAVISRHSLSAGLVGLSVSYSLQVTTYLNWLVRMSSEMETNIVA 1145
                                  TMH 16                TMH 17

O. niloticus    VERVKEYSDTEKEAEWKQESSSLPPGWPTKGCTDIFGFSLRYRHDLDPAIHNININGGEKVGIVGRTG 208
D. rerio        VERVKEYGDTEKEAEWKLENSNLPPGWPTAGHIEIHKFGLRYREDLELAICDISVNIAGGEKVGIVGRTG 1327
O. mykiss       VERVKEYADTPKEAPWTIEGSMLPLAWPTHGTIEMEEYGLQYRKGLDWALKGISLSIQEKEKVGIVGRTG 1264
H. sapiens      VERLKEYSETEKEAPWQIQETAPPSSWPQVGRVEFRNYCLRYREDLDFVLRHINVTINGGEKVGIVGRTG 1215
                                                                                  W-A

O. niloticus    AGKSSLTLGLFRIIEAAEGHIFIDGVDIAQLGLHDLRSRITIIPQDPVLFSGSLRMNLD---------- 267
D. rerio        AGKSSLTLGLFRIIEAAEGEIRIDGVNIADLGLHELRSRITIIPQDPVLFSGSLRMNLDPFDGYTDEEVW 1397
O. mykiss       AGKSSLALGIFRILEAAKGEIYIDGINIAQIGLHELRSRITIIPQDPVLFSGSLRMNLDPFDGYSDEEVW 1334
H. sapiens      AGKSSLTLGLFRINESAEGEIIIDGINIAKIGLHDLRFKITIIPQDPVLFSGSLRMNLDPFSQYSDEEVW 1285
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d) ABCC2 multiple alignment

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O. niloticus    NRFAKDIFTIDEAIPNSFRSWLLCFLGVLGTLFVICLATPFFAIVIIPLAVIYFFVQRFYVATSRQLRRL 70
O. mykiss       NRFAKDIFTVDEAIPQSFRSWIMCFLGVLGTLFVICLATPIFTAIIIPLAVVYYFVQRFYVASSRQLRRL 1175
D. rerio        NRFAKDIFTVDEMIPMSFRSWILCLLGVLGTLFVICLATPIFTAVVVPMAVVYYFVQRFYVATSRQLRRL 1154
M. domestica    NRFANDISTVDDTIPMSFRSWTMCFLSIISTAVMICVATPVFIVVIIPLAIIYIFVQRFYMATSRQLRRL 1161
H. sapiens      NRFAGDISTVDDTLPQSLRSWITCFLGIISTLVMICMATPVFTIIVIPLGIIYVSVQMFYVSTSRQLRRL 1151
                                          TMH 15

O. niloticus    DSVSRSPIYSHFGETVSGLSVIRAYKHQDRFLKHNEVTIDENLKSVYPWIVSNRWLAIRLEFVGNLVVFF 140
O. mykiss       DSVSRSPIYSHFGETVSGLSVIRAYGHQDRFLKHNEKIIDENPKSVYLWIISNRWLAIRLEFLGNLVVFF 1245
D. rerio        DSVSRSPIYSHFGETVSGLSVIRAYGHQDRFLKHNEHTIDQNLKSVYPWIVSNRWLAMRLESLGNLVVFF 1224
M. domestica    DSVTKSPIYSHFSETVSGLSIIRAFEHQQRFLKHSEGIIDTNQKCVFSWIISNRWLAVRLELVGNLVVFF 1231
H. sapiens      DSVTRSPIYSHFSETVSGLPVIRAFEHQQRFLKHNEVRIDTNQKCVFSWITSNRWLAIRLELVGNLTVFF 1221
                                                                    TMH 16

O. niloticus    SALFAVTSRDSIDSGLVGLSISYALNVTQTLNWLVRMTSELETNIVAVERVSEYTELENEADWVTDTRPP 210
O. mykiss       LALLAVIARDSLDSGLVGLSISYALNVTQTLNWLVRMTSELETNIVAVERVSEYTEMENEADWVSGIRPS 1315
D. rerio        AALFAVISRDSLNSGLVGLSISYALNVTQTLNWLVRMTSELETNIVAVERVREYAEIQNEAPWVTSVRPP 1294
M. domestica    SALLIVIYRDNLKGDMVGLVLSNALNITQTLNWLVRMTSELETNIVSVERINEYIKVKNEAPWLLEKRPP 1301
H. sapiens      SALMMVIYRDTLSGDTVGFVLSNALNITQTLNWLVRMTSEIETNIVAVERITEYTKVENEAPWVTDKRPP 1291
                                  TMH 17

O. niloticus    QQWPEAGRVQFENYKVRYRPELDLVLHGITCDIDSTEKIGIVGRTGAGKSSLTNCLF---------- 267
O. mykiss       EKWPEAGRLRFENFKVRYRPELDLVLHGITCDIDSTEKIGIVGRTGAGKSSLTNCLFRIIEAAEGRILID 1385
D. rerio        DDWPSAGNIRFEDYKVRYRPELELVLHGVTCDIQSTEKIGIVGRTGAGKSSLTNCLFRIVEAADGRILID 1364
M. domestica    DNWPSKGEIRFTDYKVRYRPELDLILHGITCNIEGTEKIGVVGRTGAGKSSLTNCLFRILEAAEGQITID 1371
H. sapiens      PDWPSKGKIQFNNYQVRYRPELDLVLRGITCDIGSMEKIGVVGRTGAGKSSLTNCLFRILEAAGGQIIID 1361
                                                                  W-A
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e) ABCG2 multiple alignment

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O. niloticus    ----IGLNGIMKPGLNAIMGATGSGKSSFLDVLAARKDPAGLTGEVLIDGAPQPPNFKCLSGYVVQDDVV 66
O. mykiss       KDILIDLNGLMKPGLNAIMGATGSGKSSFLDVLAARKDPAGLAGEVLMDGAPQPPNFKCLSGYVVQDDVV 131
D. rerio        KNILIGLNGIMKPGLNAILGATGSGKSSFLDVLAARKDPAGLSGEVLIDGAPQPPNFKCLSGYVVQDDVV 125
S. salar        REILVDLNGIMRPGLNAILGPTGSGKSSFLDVLAARKDPSGLSGEVLIDGAPQPPNFKCLSGYVVQDDVV 139
H. sapiens      KEILSNINGIMKPGLNAILGPTGGGKSSLLDVLAARKDPSGLSGDVLINGAPRPANFKCNSGYVVQDDVV 130
                                  W-A

O. niloticus    MGTLTVRENFTFSAALRLPSSISQKEKKAKVDRLIKELGLGRVADSRVGTQLIRGISGGERKRTNIGMEL 136
O. mykiss       LGTLTVRENFRFSAALRLPSSVSQKEKEDKVNRLITELGLTKVADSRVGTQLIRGISGGERKRTNIGMEL 201
D. rerio        MGTLTVRENLRFSAALRLPKSIKQREKDEKVERLIQELGLSKVADSRVGTQLIRGVSGGERKRTNIGMEL 195
S. salar        MGTLTVRENLRFSAALRLPRSVPQKEKEARVNDLITELGLTKVADAKVGTQMIRGISGGERKRTNIGMEL 209
H. sapiens      MGTLTVRENLQFSAALRLATTMTNHEKNERINRVIQELGLDKVADSKVGTQFIRGVSGGERKRTSIGMEL 200
                                                                  C-motif

O. niloticus    IIDPPVLFLDEPTTGLDASTANSVLLLLKRMANSGRTIILSIHQPRYTIYRLFDSLTMLVNGHQVYHGPA 206
O. mykiss       IIDPPVLFLDEPTTGLDASTANSVLLLLKRMSSHGRTIILSIHQPRYSIFRLFDSLTLLVSGKQVYHGPA 271
D. rerio        IIDPPVLFLDEPTTGLDASTANSVLMLLKKMGNNGRTIILSIHQPRYSIYRLFDSLTLLVGGKLVYHGPA 265
S. salar        IIDPSVLFLDEPTTGLDASTANSVLLLLKRMANQGRTIIMSIHQPRYSIYRLFDSLTLLVSGKQVYHGPA 279
H. sapiens      ITDPSILFLDEPTTGLDSSTANAVLLLLKRMSKQGRTIIFSIHQPRYSIFKLFDSLTLLASGRLMFHGPA 270
                      W-B

O. niloticus    RSALEYFSDIGYT------------------------------------------------------------ 219
O. mykiss       QSALDYFSNIGYTCPPHNNPADFFLDVINGDSTSIAFDRIKEADDSDPDRVTSSKQNIEDHLVQEYRGSQ 341
D. rerio        QDALSYFSQIGYTCPPHNNPADFFLDVINGDSSAVTLNKLY--EEVDQDQLSSSLKGIEDRLVEEYQRSS 333
Salar           QNALDYFADIGYACEAHNNPADFFLDVINGDSTATAMNKIQ-GEDIDFEELSGSRQTIEERLVEEYRNCS 348
H. sapiens      QEALGYFESAGYHCEAYNNPADFFLDIINGDSTAVALNREEDFKATEIIEPSKQDKPLIEKLAEIYVNSS 340
```

Fig. 1 (*continued*).

Samples were run in duplicate. No template controls were run to exclude contamination and the formation of primer dimers. To determine the efficiency of the PCR reactions (Table 2), standard curves were made for all the genes, with 6 serial dilutions of the template (concentrations range from 0.05 to 50 ng/μL), and the slopes and regression curves were calculated. Quantification of the expression of the genes in study, ABCB1b, ABCB11, ABCC1, ABCC2, ABCG2, CYP1A and GSTα, after exposures to BaP, was done by normalization against the reference gene (18S RNA). The ΔCt values were calibrated against the control ΔCt, and the relative expression of the target genes was calculated by the $2^{-\Delta\Delta Ct}$ method, considering efficiencies close to 100% (Livak and Schmittgen, 2001). For the purpose of inter-gene comparison throughout one tissue, relative quantification was used as a method of choice. Target genes were normalized to the reference gene (18SRNA), according to the equation,

$MNE = E_{ref}^{Ct(ref,mean)} / E_{target}^{Ct(target,mean)}$, described in detail by Simon (2003), where MNE stands for mean normalized expression; E_{ref} is the efficiency of reference gene; E_{target} is target gene efficiency; $Ct_{ref,mean}$ is the mean CT value of the reference gene; and $Ct_{target,mean}$ stands for the mean Ct value of the target gene. Tissues from control animals were used, and results were presented as target gene expression relative to reference gene expression multiplied by 10000.

2.8. Statistical analysis

Treatment effects were evaluated by means of a one-way ANOVA, followed by a multiple comparision test (Tukeys test) at a 5% significance level. Some data had to be log or square root transformed in order to fit ANOVA assumptions. All tests were performed using the software Statistica 7 (Statsoft, Inc.).

2.9. Ethics statement

The animals used in the research that is described in this paper were treated in accordance with the Portuguese Animals and Welfare Law (Decreto-Lei nº 197/96) approved by the Portuguese Parliament in 1996. Institutional animal approval by CIIMAR/UP and DGV was granted for this study.

3. Results

3.1. Identification of ABC transporters related genes

Based on the degrees of homology with the same genes in mammals and other fish species, we were able to identify partial gene sequences for ABCB1b, ABCB11, ABCC1, ABCC2 and ABCG2 in Nile tilapia.

A 922 bp sequence similar to ABCB1b, a 816 bp sequence similar to ABCB11, a 804 bp sequence similar to ABCC1, a 805 bp sequence similar to ABCC2 and a 659 bp sequence similar to ABCG2 were identified. Sequences were submitted to GenBank and given the accession numbers provided in Table 1. Analysis performed using blastx alignments, showed high degrees of homology of the identified transcripts with other fish and mammal species, including human (Table 1). Multiple alignments of the deduced amino acid (aa) sequences in Nile tilapia with other fish species and human are shown in Fig. 1. In the partial aa sequences of ABCB11 and ABCG2 the following highly conserved regions were identified: Walker-A, Walker-B and C-Motif (Fig. 1b and 1e). Topology analysis revealed that partial aa sequence of ABCB11 and ABCG2 covered almost completely a nucleotide binding domain (NBD 2 for ABCB11 — Fig. 2b, and NBD 1 for ABCG2 — Fig. 2e). The Walker A region was also identified in the partial aa sequences of ABCC1 (Fig. 1c) and ABCC2 (Fig. 1 d), and according to the topology predictions, the identified sequences partially covered membrane spanning domain 2 (MSD2) and NBD2, for both ABCC1 (Fig. 2c) and ABCC2 (Fig. 2 d). No highly conserved regions were identified in the ABCB1b partial aa sequence (Fig. 1a), and topology analysis revealed that ABCB1b sequence covered almost completely the MSD2 (Fig. 2a). Results of the phylogenetic analysis revealed that sequences identified in Nile tilapia are evolutionarily closer to the same genes in other fish species, than to mammals (Fig. 3).

3.2. Quantification of ABCB1b, ABCB11, ABCC1, ABCC2, ABCG2, CYP1A and GSTα mRNA expression in liver, gill and proximal intestine of Nile tilapia, after BaP exposures

3.2.1. ABC transporters, CYP1A and GSTα mRNA expression in the different tissues

Relative quantification was used for comparison of different genes mRNA expression within each target tissue, and results are given in Fig. 4 (a–c). The highest levels of ABCB1b mRNA relative expression were found in proximal intestine (155-fold), while in liver expression was about 9 times lower; oppositely, ABCB11 mRNA expression was about 8 times higher in liver (26.2-fold) than in proximal intestine (3.4-fold). Neither ABCB1 nor ABCB11 mRNA expression were detected in gill. ABCC1 mRNA expression increased from liver (8.6-fold), to proximal intestine (14.1-fold) and to gill (36.9-fold). Liver and gill showed similar levels of ABCC2 mRNA expression (15.1 and 8.9-fold, respectively) while in proximal intestine expression was about 10 times higher (104.1-fold). ABCG2 was about 3 times more expressed in proximal intestine (23.5-fold) than in liver and gill (7.9-fold). CYP1A mRNA was much more expressed in liver and gill (3183 and 3462-fold, respectively), than in proximal intestine, where its expression was about 17 times lower (199.2-fold). The highest mRNA expression of GSTα was seen in gill (1842-fold). In proximal intestine and liver its expression was 20 and 100 times lower (86 and 19-fold, respectively). Considering each tissue individually, in liver, CYP1A mRNA expression was different ($p<0.05$) from all ABC transporters and GSTα, and ABCB1b and ABCB11 were the most expressed transporters (Fig. 3a). In gill, CYP1A and GSTα showed a markedly higher expression than ABC transporters, and ABCC1 was the highest expressed transporter (Fig. 4b). In proximal intestine, results showed similar expressions of ABCB1b, ABCC2 and CYP1A, while ABCB11, ABCC1, ABCG2 and GSTα mRNA expression was lower (Fig. 4c).

3.2.2. Water exposure to BaP

ABC transporters, CYP1A and GSTα mRNA expression levels after water exposure to different BaP concentrations are displayed in Fig. 5 (a–f).

Relative mRNA expressions of ABCC2 (in gill) and ABCG2 (in liver and proximal intestine) were up regulated after BaP exposure, with significant differences to control animals. Gill mRNA ABCC2 fold induction over control was dose-dependent, ranging from 6 (in 10 μg/L) to 16 (in 50 μg/L) (Fig. 5c). Similar results were seen in intestinal ABCG2 mRNA expression, where a maximum 7-fold increase was detected in animals exposed to 50 μg/L of BaP. In liver, ABCG2 mRNA expression had a maximum fold increase of 2 after exposure to 25 μg/L (Fig. 5 d). ABCB1b followed a pattern of increasing mRNA expression in both liver and proximal intestine as the concentration of BaP rose (Fig. 5a), even though without statistical support. CYP1A mRNA was significantly up-regulated after waterborne BaP exposure in all three tissues (Fig. 5e). In liver, animals exposed to 50 μg/L of BaP showed a relative 5-fold induction in CYP1A mRNA expression. In gill and proximal intestine, induction of CYP1A mRNA was seen at all BaP concentrations, with relative increases of 14 and 19-fold (at 10 μg/L), 11 and 81-fold (at 25 μg/L) and 35 and 155-fold (at 50 μg/L), respectively for gill and proximal intestine. No significant differences were seen in GSTα mRNA expression in all three tissues, although an increasing pattern with BaP concentration was patent in gill (Fig. 5f).

Expression of CYP1A was highly correlated with ABCC2 mRNA in gill ($r = 0.99$, $p = 0.002$) and with ABCG2 mRNA expression in

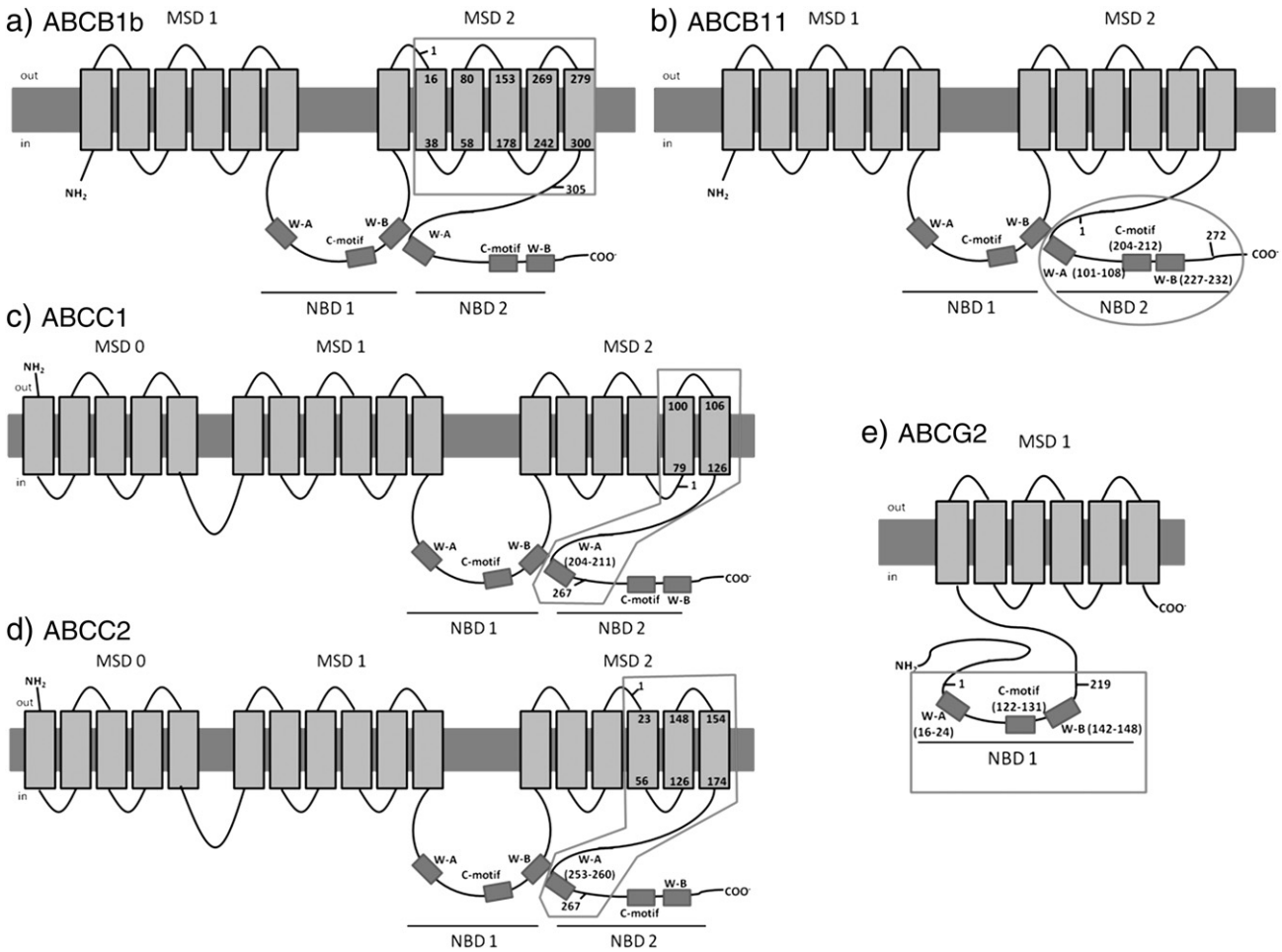

Fig. 2. Predicted topologies of Nile tilapia partial ABCB1b (a), ABCB11 (b), ABCC1 (c), ABCC2 (d) and ABCG2 (e) proteins with membrane spanning domains (MSDs) and nucleotide binding domains (NBDs) according to the Polyphobius algorithm. In NBDs, highly conserved regions of the ABC transporters superfamily are indicated: W-A (Walker A), C-motif (Signature motif) and W-B (Walker B). Regions of partial ABC transporters aa sequences identified in this study are enclosed in red frame.

intestine ($r = 0.99$, $p = 0.05$). Both in gill and proximal intestine, high CYP1A correlations matched the ABC transporters that showed dose-dependent mRNA up-regulations after BaP waterborne exposure. GSTα and ABCC2 mRNA expressions were highly correlated (0.97, $p = 0.004$) in gill tissue. Biliary BaP metabolites (measured as Fluorescent Aromatic Compounds — FACs), 7-ethoxyresorufin *O*-deethylase (EROD) and glutathione *S*-transferase activity in liver, gill and intestine from the same animals were previously determined in our laboratory (results published in Costa et al., 2011). Biliary BaP metabolites were significantly correlated with liver CYP1A mRNA ($r = 0.91$, $p = 0.004$), gill ABCC2 mRNA ($r = 0.93$, $p = 0.022$), gill CYP1A mRNA ($r = 0.95$, $p = 0.013$) and gill GSTα mRNA ($r = 0.90$, $p = 0.036$). EROD activity and CYP1A mRNA correlations were $r = 0.95$ in liver ($p = 0.014$), $r = 0.76$ in gill ($p = 0.131$) and $r = 0.86$ in proximal intestine ($p = 0.062$).

3.2.3. Dietary exposure to BaP

Gene expression levels of ABCB1b, ABCC1, ABCC2, ABCG2, CYP1A and GSTα of animals from dietary exposure to 100 and 200 μg of BaP/g of food are displayed in Fig. 6 (a–f).

In this exposure route, high variability between animals of the same treatment was seen in mRNA expression levels of the ABC transporters. Unexpectedly, expression of ABCC1 (Fig. 6b) and ABCG2 (Fig. 6 d) in proximal intestine of solvent exposed animals was higher than the levels seen in the remaining treatments. Gill of animals exposed to 100 μg of BaP/g of food had the highest levels of ABCC2 mRNA expression (Fig. 6c). Moreover, although without significant differences to control groups, ABCB1b gene expression in liver increased in animals exposed to 100 and 200 μg of BaP/g of food (Fig. 6a). No significant changes were seen in GSTα mRNA expression after Bap exposure (Fig. 6f).

In this assay, the most significant result was the induction of intestinal CYP1A mRNA in dietary exposed animals (Fig. 6e), seen after exposure to 100 and 200 μg of BaP/g of food (maximum of 13-fold increase in the latter BaP concentration). Biliary BaP metabolites (measured as FACs), EROD and GST activities in liver, gill and intestine from the same animals were previously determined in our laboratory (results published in Costa et al., 2011). Intestinal CYP1A mRNA expression was highly correlated with EROD activity ($r = 0.95$, $p = 0.048$) and biliary BaP metabolites ($r = 0.97$, $p = 0.025$).

4. Discussion

In this study, expressional changes of ABC transporters (ABCB1b, ABCB11, ABCC1, ABCC2 and ABCG2), CYP1A and GSTα were determined upon waterborne and dietary BaP exposures in *O. niloticus*. After the identification of the target partial sequences in this specie, relative mRNA expression was measured in tissues involved with the captation and excretion of pollutants and/or its metabolites (liver, gill and proximal intestine).

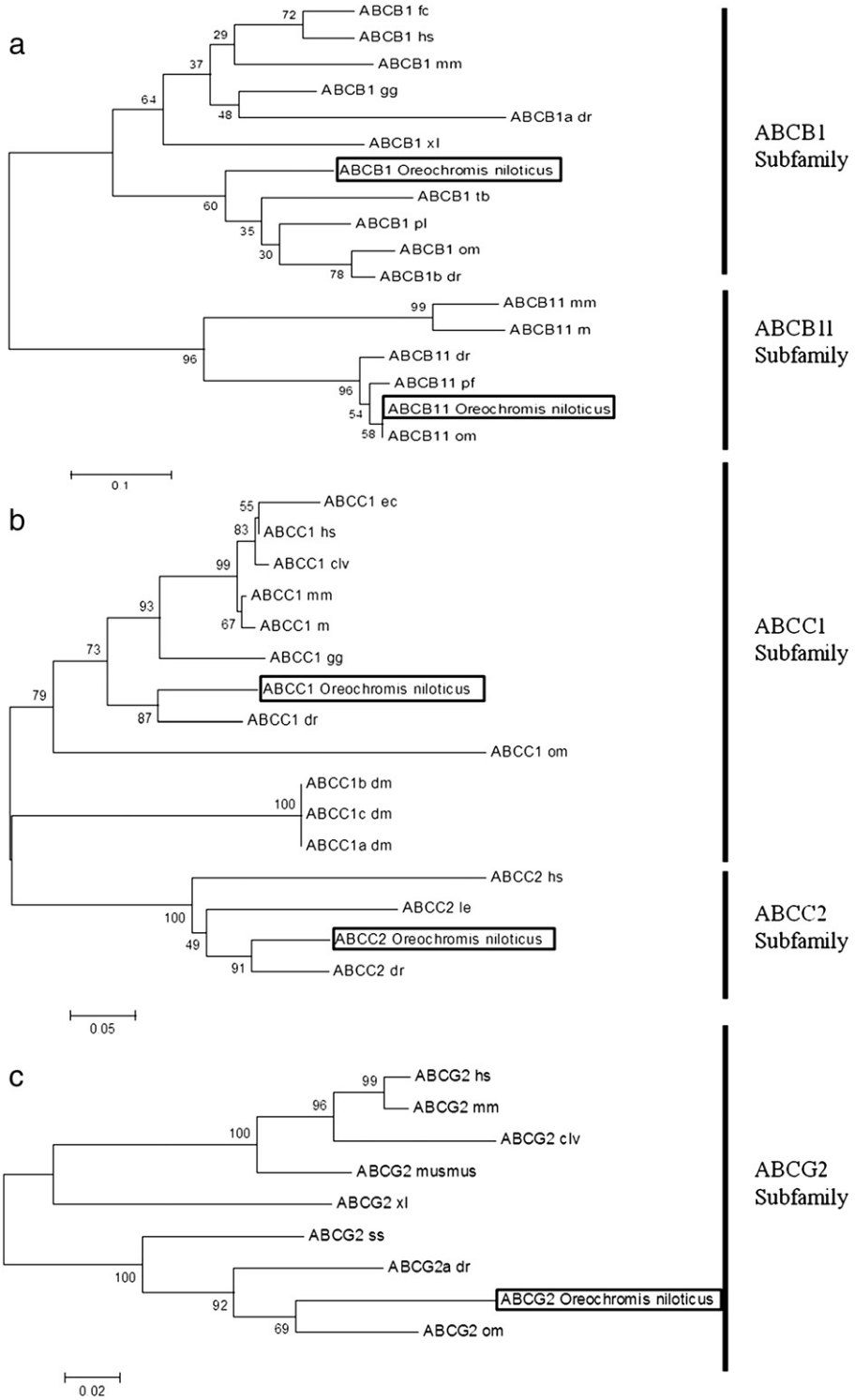

Fig. 3. Phylogenetic trees based on the multiple alignments (Clustal W) of closely related proteins from ABCB, ABCC and ABCG subfamilies found in other species. Evolutionary history was inferred using neighbor-joining method and the percentage of concordance based on 1000 bootstrap iterations is shown at the nodes. a) ABCB subfamily: ABCB1 fc — *Felis catus* NP001164535.1, hs — *Homo sapiens* NP000918.2, mm — *Mus musculus* NP035205.1, gg — *Gallus gallus* NP990225.1, dr — *Danio rerio* a ENSDARG00000021787, xl — *Xenopus laevis* NP001081394.1, tb — *Trematomus bernachii* ACX30417.1, om — *Oncorhynchus mykiss* AAW56424, dr — *D. rerio* b ENSDARG00000010936, pl — *Poeciliopsis lucida* ADQ20481.1; ABCB11 mm — *M. musculus* NP066302.2, rn — *Rattus norvegicus* NP113948.1, dr — *D. rerio* XP001923538, pf — *Platichthys flesus* CAC86593.1, om — *O. mykiss* NP001118128.1; b) ABCC subfamily: ABCC1 ec — *Equus caballus* NP001075232, hs — *Homo sapiens* NP004987.2, clv — *Canis lupus vulgaris* NP001002971.1, mm — *M. musculus* NP032602, rn — *R. norvegicus* NP071617, gg — *G. gallus* NP001012540.1, dr — *D. rerio* XP001341895.3, om — *O. mykiss* NP001161802.1, dm — *Drosophila melanogaster* NP723772.2-a, NP609591.2-b, NP995699.1-c; ABCC2 hs — *H. sapiens* CAB45309.1, le — *Leucoraja erinacea* AAL92112.1, dr — *D. rerio* XP002664118; c) ABCG subfamily: ABCG2 hs — *H. sapiens* AAH92408.1, mm — *Macaca mulatta* NP001028091.1, clv — *Canis lupus vulgaris* NP 001041486, musmus — *M. musculus* NP036050.1, xl — *Xenopus laevis* NP001091141, ss — *Salmo salar* NP001167126.1, dr — *D. rerio* NP001036240.1, om — *O. mykiss* NP 001118155.1. Tree was designed using Mega 4.0.2 software.

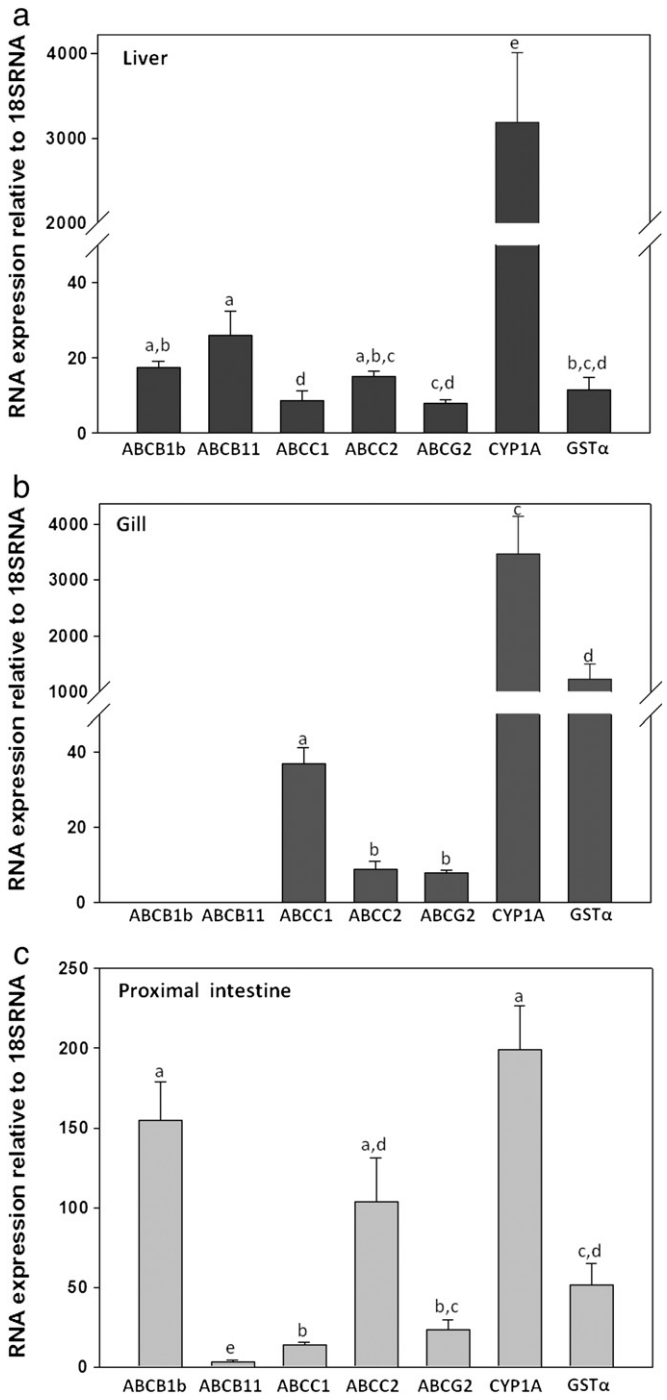

Fig. 4. Relative mRNA expression of ABC transporters, CYP1A and GST α in Nile tilapia liver (a), gill (b) and proximal intestine (c). Neither ABCB1b nor ABCB11 mRNA expression was detected in gill. 18SRNA mRNA expression was set to 10000 in all tissues. Different letters denote significant differences ($p<0.05$) between genetic expression of target genes (ABCB1b, ABCB11, ABCC1, ABCC2, ABCG2, CYP1A and GSTα) within the same tissues. Results are presented as mean ± SE ($n=4$).

4.1. Identification and tissue distribution of ABC transporters, CYP1A and GSTα

Molecular data on Nile tilapia obtained in this study, demonstrated that, with exception of ABCB1b, all the remain deduced aa sequences of the target ABC transporters (ABCB11, ABCC1, ABCC2 and ABCG2), have at least one of the classical features of this protein family, C-Motif (LSGGQ), Walker A (GxxGxGKS/T, where x equals any aa) and/or Walker B (xxxxD, where x equals hydrophobic residues), cytoplasmatic highly conserved amino acid sequences that can be found in all vertebrate ABC proteins (Hyde et al., 1990). Topology analysis (Fig. 2) revealed that partial aa sequences for the ABC transporters identified in this study are analogous to what has been described for other species (Lage, 2003; Leslie et al., 2005). Moreover, the high degree of homologies with other fish and mammals seen after multiple alignments (Fig. 1), and clustering of the sequences with other fish species in phylogenetic analysis (Fig. 3), consequently demonstrate the presence of the genes for the ABC transporters in Nile tilapia. In the case of ABCB1, two copies of ABCB1 gene can be found in zebrafish genome, called ABCB1a and ABCB1b (Annilo et al., 2006). Recently, a novel ABCB1 isoform was identified in trout and named ABCB1b (Fischer et al., 2011). Results show that Nile tilapia sequence has higher degree of identity with zebrafish ABCB1b (ABCB4), and trout ABCB1a, than to zebrafish ABCB1a (Fig. 3). Thus, based on the similarities with the isoforms originally named in zebrafish, Nile tilapia ABCB1 identified in this study should be of type b. Regarding ABCG2 gene, four isoforms (from a to d) are described in the zebrafish genome (Annilo et al., 2006). Our results have shown that ABCG2 identified in Nile tilapia has higher degree of homology with zebrafish ABCG2a (Fig. 3).

In this study, relative mRNA expression of the different ABC transporters, CYP1A and GSTα in liver, gill and proximal intestine of *O. niloticus* was assessed. Only a few studies have described the distribution pattern of ABC transporters in aquatic organisms, namely in different tissues of trout (Zaja et al., 2008a; Zaja et al., 2008c; Loncar et al., 2010), zebrafish (Long et al., 2011a), little skate (Cai et al., 2003), shipshead meadow (Hemmer et al., 1998) and *Poecilia reticulata* (Hemmer et al., 1995). The distribution pattern of ABCB1b, ABCB11, ABCC1, ABCC2 and ABCG2 found in Nile tilapia follows the ones described in those studies, and is also in agreement with tissue distribution pattern determined in mammals (Flens et al., 1996; Keppler and Konig, 1997; Gerloff et al., 1998; Mottino et al., 2000; Maliepaard et al., 2001; Brady et al., 2002), with highest expression levels of ABC transporters found in important physiological/pharmacological barriers (Szakács et al., 2008).

ABCB1b and ABCB11 mRNA expression was not detected in gill, and these results are in agreement to what is described in the literature for other fish species (Zaja et al., 2008a; Loncar et al., 2010). Zaja et al. (2008a) suggested that the high blood content in the gills could potentially mask the gene expression in specific cells types, such as gill epithelial cells, and indicated primary gill epithelial cells as a more adequate model for studying ABC transporters in gills. In subsequent works, ABCB1 and ABCB11 were detected at low levels in a gill cell line in trout (Fischer et al., 2011). ABCB11 mRNA tissue distribution pattern observed in this study, with almost exclusive expression in liver (very low expression was seen in intestine and no expression in gill), is in agreement with its described function of transporter of bile salts from hepatocytes into the bile (Gerloff et al., 1998). Similarly to what has been described for mammals and other fish species, this study has shown that, also in Nile tilapia ABC transporters have distinct mRNA expression patterns in different tissues. In mammals, these different patterns of expression have been shown to be function related (Szakács et al., 2008). Therefore, this might also be true for Nile tilapia, although more research on this matter remains necessary, namely at a post-transcriptional level and on cellular localization of ABC transporters in fish. However, this kind of approach is still difficult to achieve since, with exception of ABCB1, no functional antibodies are available for ABC transporters in fish species.

Higher levels of CYP1A mRNA expression were seen in liver and gill when comparing to the proximal intestine, with gill showing slightly higher levels of CYP1A mRNA expression than liver (Fig. 4). This data is in agreement with previous results reported in *Salmo salar*, where gill tissue had highest basal levels of CYP1A mRNA,

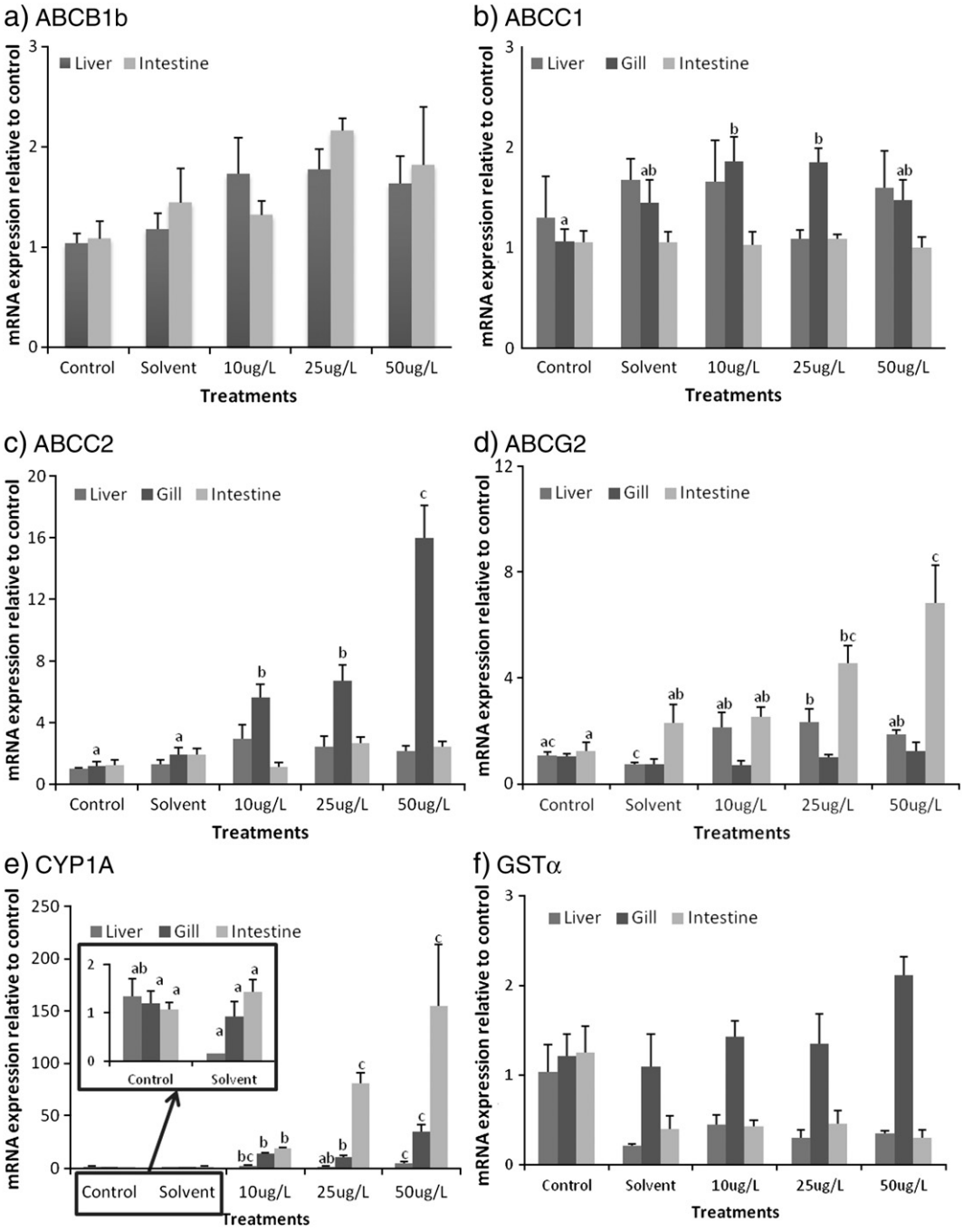

Fig. 5. Relative mRNA expression of ABCB1b (a), ABCC1 (b), ABCC2 (c), ABCG2 (d), CYP1A (e) and GSTα (f) in liver, gill and proximal intestine of animals exposed to waterborne BaP. ABCB1b mRNA expression was not detected in gill. Insert in (e) refers to detailed CYP1A mRNA expression in control and solvent. Expression was quantified by qRT-PCR, and fold changes to control were determined using the $2^{-\Delta\Delta CT}$ method. Results are given as mean ± SE (n = 4). Different letters denote significant differences ($p<0.05$) between treatments within the same tissue.

than liver, kidney and brain (Rees et al., 2003). Considering that mRNA CYP1A expression found in tilapia tissues is well correlated with the levels of CYP1A activity measured in the same animals in a previous study (Costa et al., 2011), the higher basal levels of steady state CYP1A mRNA found in gill tissue may reflect the biotransformation function of gills, when the exposure to contaminants is mainly through water (Levine and Oris, 1999; Rees et al., 2003). Additionally, major CYP1A expression and/or activity typically found in liver, is consistent with the role of this organ in xenobiotic metabolism and excretion (Levine and Oris, 1999; Sarasquete and Segner, 2000), whose overall contribution to detoxification is well accepted. GSTα mRNA expression was considerably higher in gill, than in liver and intestine (Fig. 4). GST sequences have been isolated and characterized in several fish species, such as plaice (Leaver et al., 1993; Martinez-Lara et al., 2002), large-mouth bass (Doi et al., 2004), red-seabream (Konishi et al., 2005), mangrove killifish (Lee et al., 2005), river pufferfish (Kim et al., 2010), common carp (Fu and Xie, 2006) and bighead carp (Li et al., 2010). The majority of these works reported high hepatic expression of several GST isoforms, indicating that different GST isoforms are associated with hepatic detoxification of xenobiotics (Kim et al., 2010). Moreover, high intestinal levels of GSTs have also been reported in fish (Kim et al., 2010; Li et al., 2010) and mammals (Landi, 2000), indicating a role of this

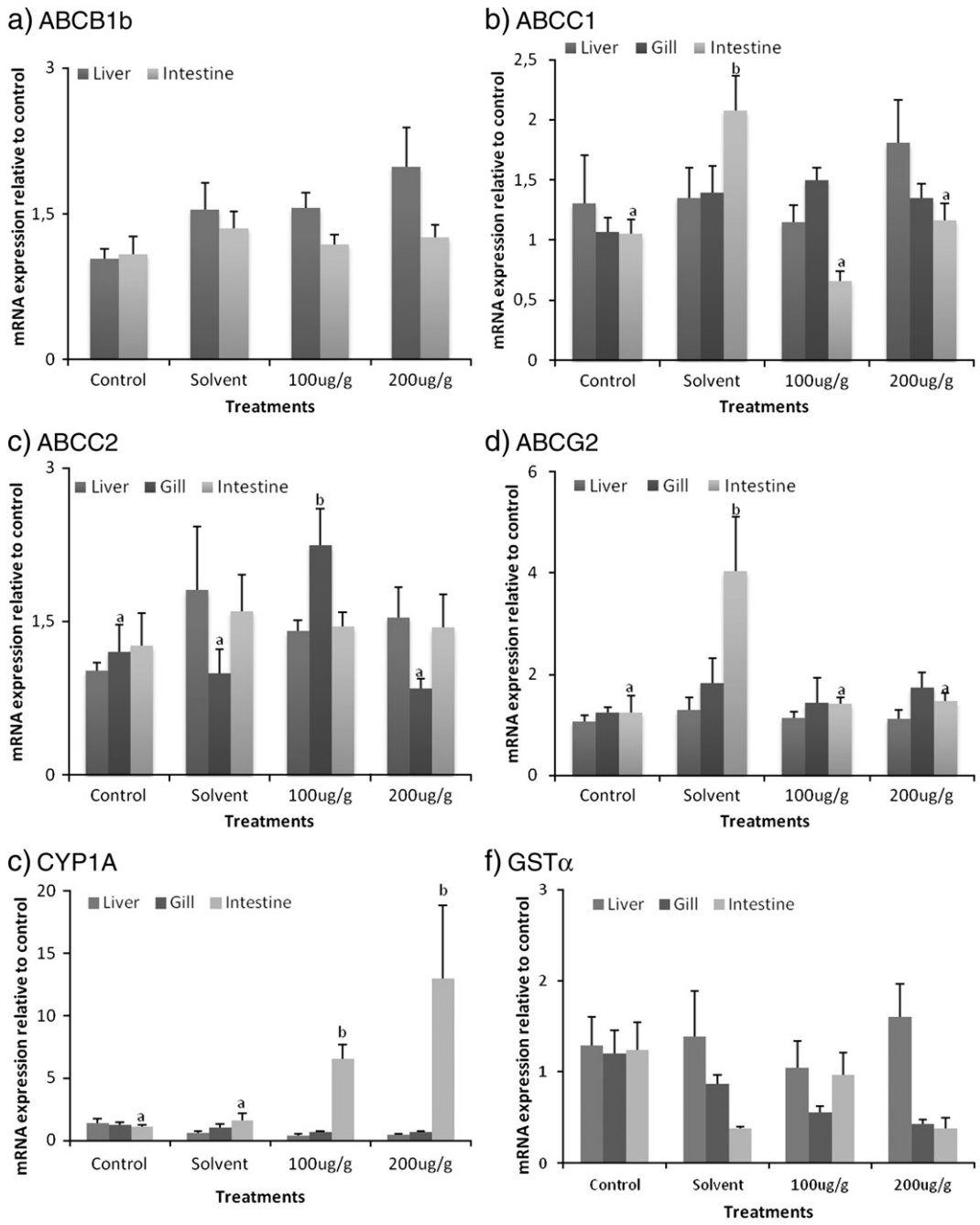

Fig. 6. Relative mRNA expression of ABCB1b (a), ABCC1 (b), ABCC2 (c), ABCG2 (d), CYP1A (e) and GSTα (f) in liver, gill and proximal intestine of animals exposed to dietary BaP. ABCB1b mRNA expression was not detected in gill. Expression was quantified by qRT-PCR, and fold changes to control were determined using the $2^{-\Delta\Delta CT}$ method. Results are given as mean ± SE (n = 4). Different letters denote significant differences ($p<0.05$) between treatments within the same tissue.

tissue in xenobiotic breakdown. Studies that compared GSTα expression in fish tissues indicated that this specific isoform has higher expression in liver and intestine than gill in bighead carp (Li et al., 2010) and river pufferfish (Kim et al., 2010), oppositely to what was found in Nile tilapia. Higher GSTα mRNA levels in gill reinforce the idea that gills also display an important role in xenobiotic metabolism (Jönsson et al., 2006; Nahrgang et al., 2010; Costa et al., 2011).

4.2. ABC transporters CYP1A and GSTα transcriptional responses upon BaP exposure

After exposure to BaP, mRNA levels of some of the studied ABC transporters, and of CYP1A were up-regulated in Nile tilapia. The most significant results were seen after water exposure, with increased mRNA expression of ABCC2 (in gill), ABCG2 (in liver and proximal intestine) and CYP1A (in liver, gill and proximal intestine). Although without significant differences, hepatic and intestinal ABCB1b and gill GSTα mRNA showed a tendency to increase in animals exposed to waterborne BaP. In dietary assays only intestinal CYP1A mRNA was significantly up-regulated in BaP exposed animals.

A small number of studies have addressed the modulation of ABC transporters in liver of fish species, and an increase of the hepatic transcripts of ABCC2 and ABCG2 in killifish exposed to PAHs (Paetzold et al., 2009) and of hepatic mRNA ABCC2 in zebrafish and Antartic fish exposed to heavy metals (Zucchi et al., 2010; Long et al., 2011b) has been reported. Our results follow the pattern of expression seen in those studies, since ABCC2 and ABCG2 were the transporters with more pronounced changes to BaP exposure.

CYP1A mRNA up-regulation in liver, gill and proximal intestine of water exposed animals, is consistent to what is described in the literature (Van Veld et al., 1997; Ortiz-Delgado et al., 2005; Ortiz-Delgado et al., 2008). Correlations between CYP1A mRNA and CYP1A catalytic activity (EROD) measured in a previous study performed in our laboratory using the same animals (Costa et al., 2011), were high for all tissues, although only significant in the liver ($r=0.95$, $p=0.014$), providing evidence that an increase in mRNA expression of CYP1A reflects an increase in protein functionality. In Costa et al., 2011, we have demonstrated that waterborne BaP exposure results in high rates of metabolism (by CYP1A enzymes) in liver, gill and intestine, and that total BaP metabolites measured in bile are correlated with CYP1A activity (measured as EROD activity), in the different tissues. Probably, BaP metabolites formed in extra-hepatic tissues are reabsorbed into the blood stream and released in the gall bladder (Kleinow et al., 1998; Costa et al., 2011). Moreover, BaP metabolites profile in plasma and bile of Nile tilapia exposed to BaP in water showed that these are mostly phase II conjugates (Rey-Salgueiro et al., 2011). In our study, high correlations were also found between total BaP metabolites (Costa et al., 2011) and 1) ABCC2 mRNA expression in gill ($r=0.93$, $p=0.022$), and 2) ABCG2 mRNA expression in intestine ($r=0.85$, $p=0.066$). Although we cannot draw any conclusions about the activity of these transporters, since no protein or functionality studies were done, mRNA up-regulation and the correlations observed with biliary BaP metabolites can suggest that, after water exposure, efflux of phase II BaP metabolites most probably occurs via ABCC2 in gill, and ABCG2 in intestine. Previous studies have reported that ABCC2 efflux of xenobiotics occurs through a co-transport mechanism with reduced glutathione (GSH) (reviewed in Leslie et al., 2005), and the significant correlation seen between GSTα and ABCC2 mRNA in gill further supports this hypothesis. Regarding the intestine, it has been hypothesized that ABC transporter proteins may be involved in the luminal directed transport of BaP metabolites in a human colon adenocarcinoma cell line and ABCB1, ABCC1 and ABCC2 transporters were ruled out as possible candidates involved in this efflux (Buesen et al., 2002; Lampen et al., 2004). However, Ebert et al. (2005) have identified ABCG2 as one of the efflux transporters of BaP conjugates formed in Caco-2 cells back into the intestinal lumen, supporting our results of ABCG2 mRNA expression in Nile tilapia intestine. Moreover, Mao (2005) reported that ABCG2 seems to preferentially transport sulfated conjugates of steroids and xenobiotics over GSH and glucoronide metabolites, which is in agreement with the lack of correlation seen between mRNA expression of this transporter and GSTα mRNA.

Despite the lack of statistical significance, we also observed an increase in ABCB1b transcriptional levels in liver and proximal intestine of water exposed animals. Up-regulation of ABCB1 in response to xenobiotics is not usually large (1–2-fold) (Smital et al., 2003), and the individual variability can be masking that response in this study. Although BaP itself has been indicated as a non-substrate for ABCB1 in mammals (Schuetz et al., 1998; Buesen et al., 2002), others indicate the opposite (Chao Yeh et al., 1992; Fardel et al., 1996) and the subject remains in discussion. Nevertheless, in a recent work by Zaja et al. (2011) no BaP interaction was seen with ABCB1 activity in a hepatoma cell line of *Poeciliopsis lucida*. In general, mRNA up-regulation of ABC transporters reported in this study could be a response to the presence of BaP and/or its metabolites. However, more work is necessary at post-transcriptional and protein levels, in order to confirm these hypothesis. Induction of ABC transporters has also been associated with a non-specific general stress response in *Mytilus californianus*, rather than to the presence of specific substrates of those transporters (Eufemia and Epel, 2000). In our study, morphological and behavioral indices (data not shown) were not altered in the animals exposed to BaP, which gives us an indication that these animals were not under stressful conditions, and that they were responding to the presence of the contaminant or its metabolites.

As previously stated, no significant changes were seen in ABC transporters after dietary exposures to BaP. Intestinal, but not hepatic, up-regulation of CYP1A transcriptional levels can indicate that animals were under BaP induced stress, and that the majority of BaP metabolism will occur at intestinal level, limiting the amount of parent compound that reaches the liver to be metabolized. This type of response has been reported at post-transcriptional levels in liver (Van Veld et al., 1987; Reynolds et al., 2003; Costa et al., 2011) and intestine (McElroy and Kleinow, 1992; James et al., 1997; Costa et al., 2011) of fish exposed to dietary PAHs. Moreover, high correlations were seen between intestinal CYP1A mRNA and intestinal EROD activity and biliary BaP metabolites (Costa et al., 2011) reinforcing the idea that mRNA expression of CYP1A reflects protein functionality, similarly to what was observed in water exposure.

Among the ABC transporters identified in this study, ABCC1 mRNA expression was not significantly altered by the presence of BaP and/or its metabolites, and was not correlated with the remaining parameters evaluated. Despite our results, much of the in vitro evidence indicates that ABCC1 has a role in the removal of toxins as glutathione, glucoronide or sulfate conjugated metabolites from cells (reviewed in Leslie et al., 2001). Additionally, ABCC1 gene induction was seen in zebrafish exposed to heavy metals (Long et al., 2011a) indicating that this transporter can be involved in heavy metal detoxification in this specie.

In dietary exposure to BaP, high variability was observed within each treatment, probably due to the fact that it is not possible to control the exact amount of food that each animal eats. This variability can be masking a possible response of ABC transporters mRNA expression, which is usually not very large, as opposed to CYP1A mRNA response (10–100 fold induction) (Epel et al., 2008; Smital et al., 2003). The induction of intestinal ABCC2 and ABCG2 mRNA expression in solvent exposed animals is an unexplainable result, as we cannot input the observed up-regulation to the presence of the solvent, since BaP exposed groups did not show the same response pattern, and were simultaneously exposed to same quantity of solvent. Moreover, this response to the solvent was only seen in these genes and, in waterborne exposure, no such effect was seen for any of the assessed genes.

5. Conclusion

In conclusion, partial mRNA sequences of ABCB1b, ABCB11, ABCC1, ABCC2 and ABCG2 were identified in *O. niloticus*, and the gene expression tissue distribution pattern of these transporters in Nile tilapia follows the ones already described in mammals and in other aquatic species. Furthermore, novel information about ABC transporters transcriptional levels after controlled exposure to BaP, an ubiquitous contaminant in aquatic environments, in liver, gill and proximal intestine is provided. Up-regulation of ABCC2 and ABCG2 transcriptional levels could be related to a possible role of these transporters in the efflux of BaP metabolites in these tissues. New information about ABC transporters provided in this work can be applied in future toxicological or physiological studies, helping to achieve a wider knowledge of the contribution of these proteins in the overall process of xenobiotic detoxification in aquatic species.

Acknowledgments

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